DEFENDING THE BARRIER

Effects of probiotics on endogenous defense mechanisms

Defending the barrier: Effects of probiotics on endogenous defense mechanisms
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Effects of probiotics on endogenous defense mechanisms

MERCK TOCH HOE STERCK

Hoe probiotica verdedigingssystemen van het lichaam beïnvloeden
(met een samenvatting in het Nederlands)

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IT IS NOT
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OF SPECIES THAT
SURVIVES, NOR
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BUT THE ONE
MOST ADAPTABLE
TO CHANGE

Charles R. Darwin

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1

General introduction

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GENERAL INTRODUCTION

"PANCREATIC DEATHS TROUBLE DUTCH"

The 24th of January 2008 was the day that the BBC headlined: "Pancreatic deaths trouble Dutch" and the day that the field of probiotic research changed indefinitely. Probiotics, which had not shown any adverse effects in numerous conditions in the past, were for the first time associated with serious adverse events. In the largest study on probiotics to that date, the Dutch Acute Pancreatitis Study Group showed that probiotic treatment was associated with a more than twofold increase in death rate in patients with severe acute pancreatitis compared with placebo treatment[1]. These results markedly intensified the debate about the place of probiotics as food supplements in the acutely ill and their safety. While the mainstream media fueled fears of consumers, suggesting that their morning ritual may be lethal, scientists bombarded scientific editorial boards with letters supporting or criticizing the trial. 'Supporters' gave credit for the way the study was conducted and analyzed, its robustness and the major impact this high–quality randomized controlled trial was going to have on administration of probiotics in the severely ill patient on intensive care units with the need for enteral feeding. Criticism mainly came from researchers from the probiotic field, who were quick to stress that organ failure before or during the first day of treatment was more frequent in the probiotic group[2], thereby attributing the unexpected results to a flawed randomization. Others blamed the probiotic mixture used[3], its dose, the mode of delivery (i.e. administered directly into the small bowel)[4] or the combination with the used fiber-rich enteral-feeding formula[5]. And while nobody forgot to emphasize the previous successes yielded with probiotic treatment, the key question: "*How is it possible that bacteria which are beneficial in less severe conditions seem to be lethal in these critically ill patients?*" was not raised, let alone answered. In a quest to find the answer, this thesis hypothesizes that probiotics can be both friend and foe and that their effects rely on timing of administration and the patient's condition.

WHO AM I?

"Who am I?" is a frequently asked question that is hardly ever answered. But as more and more evidence suggests, the question itself may need to be rephrased. There is a new and emerging field in research which underscores that it is often more informative to ask, "Who are we?"[6].

The 'we' refers to the wild abundance of bacteria and fungi that reside in the gastrointestinal tract. These unseen passengers number in the trillions and outnumber human eukaryotic cells by a factor 10, basically indicating that 'we' are 90% bacteria[7]. According to one common estimate, the human gut contains at least a

1 Besselink *et al.*,2008
2 Sand & Nordback, 2008
3 Reid *et al.*,2008
4 Marteau, 2008
5 Sand & Nordback, 2008
6 Editorial 2008
7 Shanahan, 2002

“PANCREATIC DEATHS TROUBLE DUTCH”

HOW IS IT POSSIBLE THAT BACTERIA WHICH ARE BENEFICIAL IN LESS SEVERE CONDITIONS SEEM TO BE LETHAL IN THESE CRITICALLY ILL PATIENTS?

kilogram of bacteria alone. They contribute so much to human biology that it is difficult to say where the body ends and the microbes begin. In that respect it is not surprising that they are commonly referred to as 'the forgotten organ'[8].

The understanding of our commensal microbiota remains in its infancy, which is why worldwide, multimillion dollar projects of a magnitude like 'the humane genome project' have now been started with the objective of characterizing the human microbiota in its entirety[9]. Microbiologists are understandably excited by this opportunity. So, too, are the food and pharmaceutical industries. When it comes to profitable applications to human health, the microbiome could well offer distinct advantages over the more famous genome. Human genes are notoriously difficult and risky to interfere with. But, in theory at least, the microbiome should be relatively easy to change by the selective addition or removal of bacterial species, or by altering their genetic components. This is not such a far-fetched idea. Antibiotics and 'probiotic' foods have already been shown to ease inflammatory bowel diseases in some instances. And there is increasing acceptance that certain foods, or the bacteria contained in them, can alter gut microbiota in ways that are beneficial to health in general[10]. However, before food and pharmaceutical industries can start to count their profits, a more thorough understanding of the interactions between commensals and their hosts is warranted.

INTESTINAL MUCOSAL BARRIER FUNCTION

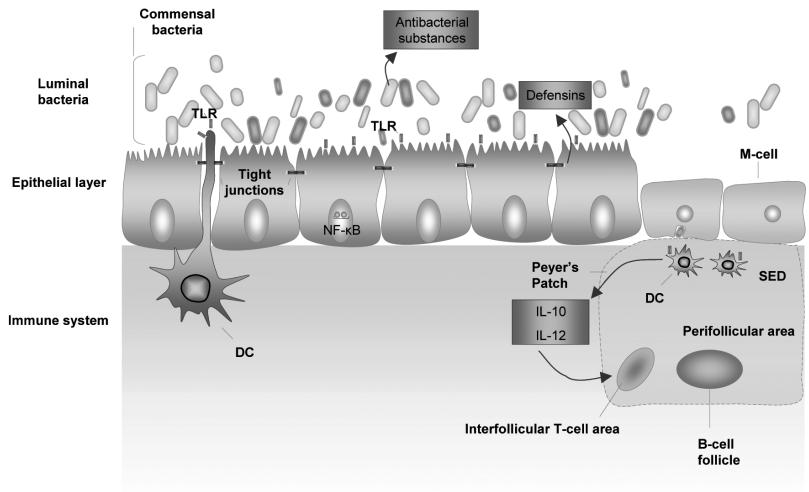
The intestinal mucosa is the largest interface between the outside world and the human internal milieu. Across a surface area that approximates the size of a soccer field[11], it is here where we prevent the highest concentration of bacteria from invading our internal environment while allowing nutrient and water absorption by a single cell layer of epithelium[12]. The ability to control the invasion of harmful content from the lumen is called *intestinal mucosal barrier function*. While the epithelial layer forms the most obvious physical boundary between inner and outer environment, the full complexity of factors that control intestinal barrier function reaches beyond the epithelium and is not fully understood. Despite a lack of knowledge of the exact mechanisms involved, mucosal barrier function can in broad terms be divided into three levels where luminal bacteria, epithelial cells, innate and adaptive immunity all work in concert to prevent potential pathogens from translocating from the lumen into the internal milieu (fig. 1).

8 Kinross *et al.*,2008
9 Turnbaugh *et al.*,2007
10 Packey & Sartor, 2009
11 Kalliomaki & Isolauri, 2003
12 Madara, 1994

FIGURE 1

Three levels of barrier function

1) Luminal commensal bacteria prevent colonization of potential pathogens by producing antibacterial substances and by forming competition for nutrients required for bacterial growth of pathogenic microorganisms. 2) Epithelial cells form the most obvious physical barrier and are interconnected by means of tight junctions. Recognition of pathogens and commensal bacteria is mediated through differential activation of Toll-like receptors (TLRs). 3) Upon recognition of potential pathogens by TLRs, epithelial cells can respond with an innate reaction to activate nuclear factor (NF)-κB. Dendritic cells (DCs) are key players in the adaptive immune response and reside primarily within the sub epithelial dome (SED) of Peyer's patches, which is covered with specialized M-cells. Once potential pathogens are recognized by DCs, these respond with the production of interleukin 12, skewing the T-cell response towards a pro-inflammatory Th1 type response.



Luminal bacteria

Colonization of the intestinal lumen is no easy feat for potential pathogens. There is an elaborate series of defenses against bacterial invasion starting with an innate distaste for the rancid smell of bacterially contaminated foods. Once within the gastrointestinal tract, bactericidal salivary lysozyme, gastric acid and pepsin, followed by bile-salts and Paneth cell-derived defensins kill most bacteria[13]. If bacteria manage to pass this evolution designed security system, they will have to face the commensal community of bacteria. The commensal microbiota possess a wide range of assets to inhibit colonization by pathogens that stretch far beyond their mere physical presence as competitive organisms, and are commonly referred to as "colonization resistance". The evolutionary arms race between pathogens and commensal bacteria resulted in great tactics to prevent potential pathogens from adherence to the epithelial surface such as production of antimicrobial substances released by bacteria to kill competitor strains, pH modification of the luminal content and the competition for nutrients required for bacterial growth of pathogenic microorganisms[14].

Epithelial layer

Throughout the intestine a single layer of epithelial cells covers the inner surface and consists of polarized cells that are joined to each other by junctional complexes. Junctional complexes consist of tight junctions, adherens junctions and desmosomes. Tight junctions are protein structures that allow selective passage of ions and small molecules, but form, in healthy subjects, a tight barrier to protein seized molecules and bacteria[15]. To make matters more complicated, the task of the epithelium is not only to keep bacteria and antigens out while absorbing nutrients, but also to allow contact between luminal contents and immune cells[16]. This occurs through limited and highly controlled uptake of antigen and bacteria. This seemingly paradoxical task is, however, crucial in the induction of targeted and protective mucosal immune responses to pathogens as well as to the development of oral tolerance to commensals and food antigens[17].

Immune system

Already at the level of the epithelial cells, invading bacteria encounter elements of the innate immune system that will inhibit them from entering the human internal environment. Epithelial cells are equipped with Toll-like receptors, which are capable of recognizing both commensal and pathogenic bacteria and their toxic products such as lipopolysaccharide. When detected by Toll-like receptors, potential pathogens are rendered harmless by direct induction of pro-inflammatory machinery within epithelial cells, leading to an immune response directed against these intruders[18].

Once past the epithelial layer, potential pathogens are still far from safe ground. The collective gut-associated lymphoid tissue is the largest immune organ in the body and much of this is organized in discrete structures, named Peyer's patches,

13 Shanahan, 2002
14 Hooper *et al.*,1999; Lievin *et al.*,2000
15 Van Itallie & Anderson, 2006
16 Gebert, 1997
17 Neutra *et al.*,2001
18 Takeda *et al.*,2003

which are in essence mucosal lymph nodes covered with specialized epithelium, follicle-associated epithelium (FAE). Unlike the surrounding villous epithelium, FAE contains microfold (M)-cells, which possess the unique ability to take up antigens and bacteria from the gut lumen through endocytosis and deliver these to antigen presenting cells and lymphocytes located in the Peyer's patches located on the basolateral side[19]. Below the epithelial layer, the adaptive immune system provides humoral and cell mediated immunity against ingested antigens and bacteria. Impressively, the adaptive immunity features the selective ability to ignore or respond to antigens, based on past encounters. Dendritic cells, which reside below the epithelial layer, are mainly responsible for the decision to either ignore or to respond to invading antigens and bacteria. Upon internalization of bacteria or antigens, dendritic cells orchestrate immune responses by instructing the T-cell population. Tolerance to food antigens, for example, results from induction of regulatory T-cells that prevent immune responses by expressing anti-inflammatory cytokines (i.e. IL-10), ensuring that the intestinal tract does not react with a full immune response to harmless antigens as the ones commonly found in foods[20]. However, when potential pathogens, recognized by differential activation of Toll-like receptors, invade the mucosa, dendritic cells are able to provoke a pro-inflammatory response by expressing cytokines (i.e. IL-12) that will generate a Th1 immune response, maximizing bactericidal activity of macrophages and recruiting immune cells to the site of infection[21]. Bacteria hate when that happens, because it is lethal.

INTESTINAL MUCOSAL BARRIER DYSFUNCTION

Given the complexity of this defensive system it is not at all surprising that in numerous pathophysiological conditions disturbances in barrier function play a major role.

Mucosal barrier function, or rather the dysfunction thereof, has been extensively studied in the context of critical illness. During periods of critical illness, the gut can be envisioned as the 'motor' of this pathophysiological state, as gut-barrier dysfunction can initiate and propagate sepsis, systemic inflammatory response syndrome and multi organ failure[22]. In a critically ill state, disruption of the equilibrium of the otherwise symbiotic three-way partnership between intestinal microbiota, epithelium, and immune system occurs. Firstly, in critically ill patients, several factors are involved in the altered composition of the intestinal microbiota, including the use of broad-spectrum antibiotics, changes in nutrient availability, gut motility, pH, osmolality and release of high levels of stress hormones, including catecholamines[23]. In fact, Freestone and colleagues (2007) showed catecholamine-induced growth of pathogens such as *Yersinia enterocolitica, Escherichia coli* and *Salmonella enterica*. Secondly, oxidative stress resulting from intestinal hypoperfusion during critical illness disrupts epithelial tight junctions[24] and induces epithelial cell apoptosis[25], both contributing

19 Gebert, 1997
20 Neutra *et al.*,2001
21 Hart *et al.*,2004
22 Clark & Coopersmith, 2007
23 Alverdy *et al.*,2003
24 Basuroy *et al.*,2006
25 Wu *et al.*,2007

to failure of the mucosal barrier. Thirdly, mucosal barrier dysfunction will provide the opportunity for luminal content (e.g. potential pathogens) to translocate through the mucosal barrier, causing a marked pro-inflammatory response, which will only further enhance the systemic inflammatory response syndrome[26].

However, one does not have to be critically ill to have an insufficient intestinal barrier function. Even under less severe conditions, such as psychological stress, a breach in barrier function can be measured[27]. The influence of psychological factors in gastrointestinal diseases has long been ridiculed by the scientific community. However, as scientific studies are providing convincing evidence that stress has a major impact on mucosal barrier function, the pendulum has finally swung. Stress and especially chronic stress (e.g. daily hassles such as traffic, financial problems, etc.) have been associated with disease exacerbation in Crohn's disease and increased numbers of relapses in ulcerative colitis patients[28]. Stress has been defined as any threat to the homeostasis of an organism. This threat can be physical, such as for instance a critically ill state, or only perceived (psychological). Regardless of the type of threat, the principal stress responses triggered and their effects on the mucosal barrier are surprisingly similar in affecting the three levels of barrier function. Firstly, evidence suggests that *Lactobacilli* respond to stress-induced changes in the intestinal physiology, such as inhibition of gastric acid release[29], alterations in gastrointestinal motility, or increased duodenal bicarbonate production[30]. These changes may result in an intestinal environment less conducive to *Lactobacilli* survival, adherence, and proliferation, causing a significant reduction in the abundance of *Lactobacilli*[31]. Secondly, and maybe most typical for stress-induced barrier dysfunction, is the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which involves the release of corticotropin-releasing hormone (CRH)[32]. CRH activates mast cells to release a large variety of pro-inflammatory mediators, e.g. histamine and TNF-α, which are known to destabilize tight junctions and to negatively affect mucosal barrier function[33]. Several studies so far have highlighted the importance of mast cells in stress-related mucosal barrier dysfunction[34]. Structural evidence for the role of mast cells in stress induced intestinal dysfunction has been obtained by studies in mast cell-deficient (Ws/Ws) rats. While their stressed wild-type littermates showed increased intestinal permeability and signs of mucosal inflammation, mast cell-deficient animals were normal with respect to epithelial function and mucosal morphology[35]. Finally, stress induces aberrant mucosal immune reactions which cause commensal bacteria to be perceived as a threat, resulting in loss of barrier function, increased penetration of bacteria into the mucosa, and increased cytokine production[36].

Taken together, the above form a possible paradigm for the pathogenesis of intestinal inflammation in that these diseases require a minimum of three conditions

26 Clark & Coopersmith, 2007
27 Soderholm & Perdue, 2006
28 Levenstein *et al.*,2000
29 Lenz & Druge, 1990
30 Lenz, 1989
31 Bailey & Coe, 1999
32 Soderholm & Perdue, 2006
33 Santos *et al.*,1999
34 Soderholm *et al.*,2002; Wallon *et al.*,2007; Yang *et al.*,2006
35 Soderholm *et al.*,2002
36 Nazli *et al.*,2004

to be present for the development of the disease. The first requirement is clearly the presence of a luminal pathogen or inciting antigen; no pathogen, no disease. The second is a seemingly misguided over exaggerated response of the immune system to these antigens. And finally, the third requirement is that the antigen must reach the immune system. This may require abnormal intestinal permeability or some other breach in the intestinal barrier.

RATIONALE FOR THE USE OF PROBIOTICS IN BARRIER DYSFUNCTION

Recognition of the beneficial effects of some bacteria has encouraged the development of probiotics as treatment for disease. Probiotics are living organisms which provide health benefits, opposed to pathogens with which most clinicians are more familiar. Probiotics have been derived from a wide range of sources, including the commensal microbiota from a particular soldier in the World War I trenches who proved resistant to dysentery (*Escherichia coli* Nissle 1917)[37], neonatal stool (*Bifidobacterium infantis*) as well as starter cultures for yoghurt (*Bifidobacterium longum*)[38].

Probiotics are, in theory, an excellent tool for restoring mucosal barrier function in patients who need it most, such as critically ill patients or patients suffering from inflammatory bowel disease (IBD). Probiotic strains and mixtures have shown effects on the three levels of barrier dysfunction and are potentially able to restore this three-way equilibrium. Direct effects within the intestinal lumen include: fortification of colonization resistance by secretion of antimicrobial bacteriocins, competitive growth[39], reduction of adherence of potential pathogens[40], degradation of pathogenic bio-films [41] and interference with the sophisticated communication systems by which potential pathogens determine to "switch on" virulence genes[42]. Furthermore, certain probiotics are known to possess anti-apoptotic properties[43] and to maintain cytoskeletal integrity, preventing disruption of tight junctions[44]. But one of the most studied and well known mechanisms of action of probiotics is their immunomodulating capacity. For instance, probiotics have shown to induce the release of anti-inflammatory cytokines such as IL-10[45], to modulate human dendritic cell function[46], and to ameliorate a pro-inflammatory response[47].

CLINICAL EVIDENCE OF PROBIOTIC EFFECTS

Despite the overwhelming load of experimental evidence suggesting that probiotics are the answer to all kinds of gastrointestinal problems, clinical evidence

37 Nissle, 1918
38 Marteau, 2000
39 Lievin *et al.*,2000
40 Sherman *et al.*,2005
41 Kim *et al.*,2009
42 Medellin-Pena *et al.*,2007
43 Yan *et al.*,2007
44 Petrof *et al.*,2004; Ait-Belgnaoui *et al.*,2006
45 Di Giacinto *et al.*,2005
46 Hart *et al.*,2004
47 Galdeano *et al.*,2007

showing the efficacy of probiotics is hard to come by and often inconclusive. Patients that could potentially benefit from probiotic treatment can broadly be divided into two main categories: firstly, patients that, on admission, are already suffering from a (gastrointestinal) illness (e.g. severe acute pancreatitis, multi trauma patients) and secondly, patients that are expected to become ill in a set period of time (e.g. patients undergoing major elective abdominal surgery or IBD patients in remission with an increased risk of relapse). The critical difference between the two being that unlike the first category of patients, the latter category provides the clinician with the opportunity to start treatment before the illness occurs, which has been shown to be advantageous when applying probiotics as a clinical treatment strategy. There is clinical evidence that probiotics can reduce the risk of relapses in IBD[48] and have a benefit as prophylactic treatment in abdominal surgery[49]. However, the most compelling clinical evidence suggesting that timing is the name of the game in probiotic treatment can be derived from a trial conducted by Sugawara *et al.* (2006), demonstrating that consecutive pre-operative and postoperative probiotic treatment is more effective in reducing postoperative infectious complications as compared to postoperative treatment alone.

In contrast to expectations and despite two smaller randomized controlled trials that showed benefits of probiotics[50] in predicted severe acute pancreatitis patients, the Dutch Acute Pancreatitis Study Group recently published a large scale multi-center randomized controlled trial which showed for the first time serious adverse effects after administration of probiotics[51]. In this study, 296 predicted SAP patients were allocated to receive either a multi species probiotic mixture, composed of 1010 *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus salivarius, Lactococcus lactis, Bifidobacterium bifidum*, and *Bifidobacterium lactis* or placebo. Treatment started within 72 hours of onset of symptoms and was given for 28 days. The incidence of infectious complications was largely comparable between the groups (probiotics *vs.* placebo, 30% *vs.* 28%); however, mortality rates were significantly higher in the probiotics group (16% *vs.* 6%) as was the incidence of bowel ischemia, which occurred in 9 probiotic treated patients compared to none in the placebo group. Interestingly, only in patients that developed multi organ failure did probiotic treatment cause an increase in intestinal-fatty-acid-binding-protein (IFABP) concentrations in urine, suggesting a deleterious effect of probiotics on the intestinal mucosa only in the most critically ill. Furthermore, despite the fact that probiotics showed an overall reduction in bacterial translocation (as measured by nitric oxide excretion in urine) in patients with organ failure, probiotics had the opposite effects and increased nitric oxide excretion, again an indication that the same probiotics may have beneficial effects in moderately ill patients and cause severe adverse events in the critically ill[52]. These results, being dramatically different from what was expected, show the need to study mechanisms of action of probiotics in critical illnesses, as probiotics can no longer be defined as 'micro-organisms with health promoting properties' without adding: 'in the right patient and at the right time'.

48 Gionchetti *et al.*,2002
49 Nomura *et al.*,2007; Rayes *et al.*,2007
50 Olah *et al.*,2002; Olah *et al.*,2007
51 Besselink *et al.*,2008
52 Besselink *et al.*,2009

WHAT DOES NOT KILL YOU MAKES YOU STRONGER

One of the difficulties in assessing the place of probiotics in clinical practice is our limited understanding of their mechanisms of action, especially their effects in patients in a critically ill state. However, with the above in mind it can be envisioned that probiotics may have a safer place in preventive applications and may not be a defendable treatment option after the onset of the critical illness, suggesting that protective effects of probiotics are dependent on timing of administration.

Despite decades of probiotic research and a vast and growing body of evidence on their mechanisms of action, the concept of probiotic strains that provide a minor toxic assault and thereby stimulate endogenous defense systems, affording better protection against future damage, is relatively new and unexplored. This would explain the seemingly paradoxical and adverse effects of probiotics in severe acute pancreatitis patients, demonstrating an increase in mortality after probiotic administration[53]. Keeping in mind that enteral probiotics may be a minor toxic burden, probiotics administered after the onset of acute pancreatitis might act as an extra burden in an already critically affected intestinal system[54], thereby doing more harm than good. The concept that probiotics enhance endogenous defense mechanisms may be a good target for research for developing preventive applications of probiotics, e.g. to more or less 'precondition' surgical patients in advance of their surgical procedure.

"IT IS NOT THE STRONGEST OF SPECIES THAT SURVIVES, NOR THE MOST INTELLIGENT, BUT THE ONE MOST ADAPTABLE TO CHANGE"

As Charles Darwin already stated; in order to survive under circumstances of stress (either psychological or physical) it is crucial to be able to adapt to stressful stimuli, to combat these threats. Owing much to the constant stream of various threats throughout evolution, mammals are equipped with rather sophisticated endogenous defense systems that allow cells to overcome stressful stimuli and that are in fact adaptable to the level of threatening signals from the environment. Two major endogenous systems that control homeostasis under stress are involved in anti-inflammatory pathways and in defenses against oxidative stress.

Anti-inflammatory defense

It should be noted that our immune system tends to overreact to any challenge, as too timid a response may be fatal. The damage associated with the inflammatory process is the price we pay for a vigilant guard. A defense mechanism that has received considerable experimental attention as a possible inflammation limiting system, is under control of peroxisome proliferator-activated receptors (PPARs), a group of 3 transcription factors originally implicated in adipocyte differentiation and glucose homeostasis. One of its isoforms (PPAR-γ) is known to exert anti-inflammatory effects by interfering with activity of inflammatory transcription factors, such as NF-κB[55], activating protein-1 (AP-1)[56], and signal transducer and activator of

53 Besselink *et al.*,2008
54 Ammori, 2003
55 Kelly *et al.*,2004
56 Yamazaki *et al.*,2007

WHAT DOES NOT KILL YOU MAKES YOU STRONGER

PRETREATMENT WITH PROBIOTICS EXERTS ITS EFFECTS IN PART THROUGH ENHANCEMENT OF ENDOGENOUS DEFENSE SYSTEMS

transcription (STAT)[57]. PPAR-γ is expressed in various tissues and cell types including intestinal tissues and dendritic cells[58] and plays a role in regulation of intestinal inflammation. Indeed, administration of synthetic PPAR-γ agonists reduces the production of inflammatory cytokines in dendritic cells[59] and ameliorates intestinal inflammation in different experimental colitis models[60] Furthermore, up-regulation of intestinal PPAR-γ expression occurs in response to stress signals such as LPS challenge[61] and stress-induced colonic inflammation[62], suggesting that the enhanced expression of PPAR-γ in the colon may occur as an adaptive and compensatory mechanism to down-regulate mucosal inflammation.

Probiotic bacteria have been shown to affect PPAR-γ, modulating intestinal epithelial inflammatory responses and barrier function[63]. For example, Ewaschuk *et al.* (2007) showed that probiotics attenuate intestinal barrier dysfunction in a LPS-induced mouse model of sepsis via PPAR-γ dependent pathways, which was proven by the fact that beneficial effects of probiotics were abolished by PPAR-γ inhibition. However, precise interactions between probiotics and this anti-inflammatory defense system are largely unknown.

Anti-oxidative defense

Free radicals, oxidative stress, and antioxidants have become commonly used terms in discussions of disease mechanisms and it is becoming increasingly more recognized that reactive oxygen species (ROS) play an important role in a wide range of diseases. The reason that overproduction of free radicals is a feature of such a broad spectrum of diseases derives from the fact that oxidative metabolism is a necessary part of every cell's metabolism[64]. And indeed, it has been found that mitochondria are the major source of free radical production. ROS have long been considered as an unwanted, reactive by-product of oxidative metabolism for the reason that radicals can initiate lipid peroxidation, protein oxidation, and DNA damage, leading to cell dysfunction and death by apoptosis or necrosis. Given the constant attack by potentially destructive, endogenously generated ROS, it is not surprising that sophisticated defense mechanisms to prevent damage and impaired function as a consequence of oxidation have evolved under the pressure of evolution[65].

Glutathione is the most abundant endogenous anti-oxidant and plays a pivotal role in preventing oxidative damage[66]. As glutathione is part of an adaptive anti-oxidative defense system, a moderate increase in intracellular ROS concentrations may paradoxically afford protection against oxidative stress *via* upregulation of oxidative defense mechanisms. In fact, its *de novo* synthesis is found to be enhanced after low dose H_2O_2[67] and is also increased by other weak oxidative agents[68].

57 Linard *et al.*,2008
58 Dubuquoy *et al.*,2006
59 Nencioni *et al.*,2002
60 Su *et al.*,1999; Desreumaux *et al.*,2001)
61 Ewaschuk *et al.*,2007
62 Ponferrada *et al.*,2007
63 Madsen *et al.*,2001; Jijon *et al.*,2004
64 McCord, 2000
65 Meister, 1995
66 Deitch *et al.*,1994
67 Ding *et al.*,2008
68 Solis *et al.*,2002

Oxygen free radicals are an essential component of the bactericidal arsenal of neutrophils. However, it should be noted that surrounding healthy host cells may be injured or even killed in the crossfire, generating an inflammatory response. Glutathione is, in effect, therefore not only an anti-oxidant defense, but also an anti-inflammatory mediator, allowing us to attenuate inflammatory responses to spare ourselves the damage associated with it[69].

There is evidence that ROS are rapidly generated by intestinal epithelium in response to commensal microorganisms, which may influence the defense against oxidative stress[70]. The involvement of probiotics in oxidative defenses, however, has yet to be elucidated.

WHO ARE WE?

Since we are the resultant of everything that has not killed us, and which thereby determined our strength, a better understanding of factors that influence our defense systems may provide more insight into who we are.

69 Biolo *et al.*,2007
70 Kumar et al.,2007

CENTRAL HYPOTHESIS AND RESEARCH QUESTIONS

The outline of this thesis is based on its central hypothesis:

"Pretreatment with probiotics exerts its effects in part through enhancement of endogenous defense systems."

To provide additional insight into this relatively unexplored combination of mechanisms by which probiotics may protect intestinal mucosal barrier function, the following main issues are addressed in this thesis:

1. Current views on mechanisms of action of probiotics and the rationale for their efficacy in barrier dysfunction (chapter 2)
2. The effects of probiotic pretreatment on the different levels of barrier function.
    a. Effects within the lumen (chapter 4, 8 and 9)
    b. Effects on epithelial barrier function (chapters 3-5 and 8-10)
    c. Effects on innate (chapter 3) and adaptive (chapter 8) immune responses
3. Extra-intestinal effects of pretreatment with probiotics.
    a. Effects on pancreatic injury (chapter 6)
    b. Effects on liver injury and its inflammatory response (chapter 7)
4. The effects of pretreatment with probiotics on endogenous defense systems
    a. Including anti-oxidative defenses (chapters 5-7)
    b. Including anti-inflammatory defenses (chapters 8-10)

To study these issues, the effects of probiotics on intestinal mucosal barrier function are examined in four experimental models of barrier dysfunction:

1) an *in vitro* model of metabolically stressed epithelial cells, designed to resemble the epithelial pathophysiology in IBD (chapter 3),
2) a rat model of critical illness, induced by induction of severe acute pancreatitis (chapters 4-8),
3) an animal model of chronic stress in which rats are submitted to water avoidance stress (chapter 9),
4) an *in vitro* model of psychological stress in epithelial cells, induced by addition of the stress hormone CRH (chapter 10).

These four models are suitable to test three levels of probiotic action, including: mucosal barrier function (table 1), extraintestinal effects (table 2) and effects on endogenous defense systems (table 3).

TABLE 1

Brief summary of methods used to study intestinal barrier function

Level of barrier function	Used methods	Chapter
Luminal effects		
Bacterial abundance	Microbiological cultures of luminal content	4
Bacterial adhesion	Immunofluorescent imaging of adherent *E. coli* Microbiological cultures of excised and washed mucosal samples	8,9
Epithelial barrier		
Mucosal permeability	Ussing experiments to determine mucosal permeability to differentially sized molecules and bacteria	5,8,9
Bacterial translocation	Microbiological cultures of extraintestinal organs	4,9
Tight junction integrity	Immunohistochemical staining of tight junctional proteins (i.e. occludin, claudin-1 and -2)	5
Epithelial cell apoptosis	Immunofluorescent TUNEL stain of ileal sections and quantification of DNA-fragmentation	5
Permeability of epithelial cell monolayers *in vitro*	Permeability of monolayers of epithelial cells to *E. coli* and differentially sized fluorescent beads. Transepithelial electrical resistance was used as index of paracellular permeability	3,10
Immune response		
Innate immunity		
Epithelial NF-κB activation	Quantification of the phosphorylated form of the inhibitor of NF-κB to indirectly determine NF-κB activation	3
Mast cell TNF-α secretion	Determination of TNF-α levels in supernatants of mast cell cultures	10
Adaptive immunity		
Dendritic cell function	Immunohistochemical assessment of dendritic cell abundance, maturation and cytokine production	8

TABLE 2

Brief summary of methods used to study extra-intestinal damage

	Used methods	Chapter
Pancreatic injury		
Histological injury score	Histological scoring of pancreatic injury	6
Pancreatic apoptotic cell death	Immunofluorescent TUNEL staining of apoptotic cells. Determination of DNA-fragmentation and caspase-3 levels	6
Oxidative stress induced pancreatic damage	Quantification of lipid peroxidation levels	6
Liver injury		
Histological injury score	Histological scoring of hepatic injury	7
Hepatic apoptotic cell death	Determination of DNA-fragmentation and caspase-3 levels	7
Oxidative stress induced hepatic damage	Quantification of lipid peroxidation levels	7
Local immune response		
Levels of pro-inflammatory mediators		
NF-κB activation	Quantification of NF-κB activation, which is an early marker of a pro-inflammatory response	6,7
IL-1β converting enzyme levels	Determination of IL-1β converting enzyme levels, which converts pro- IL-1β into its active form	6,7

TABLE 3

Brief summary of methods used to study endogenous defense systems

Defense system	Used methods	Chapter
Anti-oxidative defense		
Major endogenous anti-oxidant levels	Quantification of glutathione (GSH) levels	5-7
Endogenous anti-oxidant biosynthesis		
Availability of rate-limiting precursor	Quantification of levels of cysteine	5-7
Activity of the rate-limiting enzyme facilitating biosynthesis	Determination of the activity of glutamate-cysteine-ligase (GCL), the rate limiting enzyme in GSH biosynthesis	5-7
Gene expression of the rate-limiting enzyme facilitating biosynthesis	Quantification of mRNA levels of the two subunits of GCL (GCLc and GCLm)	5-7
Anti-inflammatory defense		
PPAR-γ activation	Quantification of nuclear PPAR-γ transcription factor	8-10
Determination of the levels of the endogenous agonist of PPAR-γ	Quantification of 15d-PGJ_2 levels, which is the endogenous ligand of PPAR-γ	9,10

2

The role of microbiota and probiotics in stress-induced gastrointestinal damage.

Probiotics, stress and barrier dysfunction

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ABSTRACT

Stress has a major impact on gut physiology and may affect the clinical course of gastro-intestinal diseases. In this review, we focus on the interaction between commensal gut microbiota and intestinal mucosa during stress and discuss the possibilities to counteract the deleterious effects of stress with probiotics.

Normally, commensal microbes and their hosts benefit from a symbiotic relationship. Stress does however, reduce the number of lactobacilli; while on the contrary, an increased growth, epithelial adherence and mucosal uptake of gram-negative pathogens, e.g. *E. coli* and *Pseudomonas*, are seen. Moreover, intestinal bacteria have the ability to sense a stressed host and up-regulate their virulence factors when opportunity knocks.

Probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host", and are mainly represented by Lactic Acid Bacteria. Probiotics can counteract stress-induced changes in intestinal barrier function, visceral sensitivity and gut motility. These effects are strain specific and mediated by direct bacterial-host cell interaction and/or via soluble factors. Mechanisms of action include competition with pathogens for essential nutrients, induction of epithelial heat-shock proteins, restoring of tight junction protein structure,

up-regulation of mucin genes, secretion of defensins, and regulation of the NF-κB signaling pathway. In addition, the reduction of intestinal pain perception was shown to be mediated via cannabinoid receptors.

Based on the studies reviewed here there is clearly a rationale for treatment of patients with stress-related intestinal disorders with probiotics. We are however far from being able to choose the precise combination of strains or bacterial components for each clinical setting.

INTRODUCTION

The gastrointestinal tract is one of the largest interfaces between the outside world and the human internal environment, which, from mouth to anus, forms a nine meter long tube with an estimated surface area between 250 and 400 m^2 or comparable in size to a tennis court[1]. The gastrointestinal tract also harbors the single largest community of immune cells[2], contains the same number of neurons as the spinal cord[3], and is the natural habitat for a large, diverse and dynamic population of microbiota that outnumber the number of eukaryotic cells in the human body by a factor 10^4. Throughout the intestine a single layer of epithelial cells is faced with the complex task of providing a barrier to potential pathogens in the lumen, while also allowing nutrient and water absorption.

Individuals suffering from chronic daily-life stressors such as loss, financial problems, unemployment, etc., will be predisposed to diseases in the gastrointestinal tract, e.g. exacerbations of irritable bowel syndrome (IBS)[5], ulcerative colitis[6] or gastroesophageal reflux disease[7]. Current evidence suggests that the stress-induced gut mucosal response is primarily mediated by neuroimmune interactions between the autonomic and enteric nervous systems and the intestinal immune system, and many studies have identified that chronic stress is associated with a decrease in systemic immune function[8]. Moreover, recent studies indicate that the consequences of chronic stress on the development of intestinal inflammation in animal models and exacerbations in patients with stress-related intestinal diseases are mediated via pronounced effects on host defense against luminal bacteria. In this article we review the current knowledge in the interaction between the intestinal microbiota and the intestinal mucosa during stress and discuss the possibilities to counteract deleterious effects of stress with probiotic treatment.

1 Kalliomaki & Isolauri, 2003
2 Brandtzaeg *et al.*,1999
3 Costa *et al.*,2000
4 Shanahan, 2002
5 Bennett *et al.*,1998
6 Levenstein *et al.*,2000
7 Naliboff *et al.*,2004
8 McEwen, 2003

WE REVIEW THE INTERACTIONS BETWEEN INTESTINAL MICROBIOTA AND THE MUCOSA DURING STRESS AND DISCUSS THE POSSIBILITIES TO COUNTERACT DELETERIOUS EFFECTS OF STRESS WITH PROBIOTICS

A STABLE COLONIZATION OF THE STOMACH AND DUODENUM IS NO EASY FEAT FOR MOST MICROBES

THE INTESTINAL MICROBIOTA

PRIMARY COLONIZATION

The fetal gut is sterile, but colonization begins once the infant passes through the birth canal. The primary colonization is influenced by the mode of delivery, environment, and type of nutrition. In the first few days the composition remains variable and develops into a more or less stable composition after the first week[9]. In breast-fed infants, the microbiota is rapidly dominated by bifidobacteria, whereas in formula fed infants the number of these bacteria may be ten times lower[10]. Furthermore, in infants born by caesarean section the establishment of a stable composition of commensal bacteria is consistently delayed[11]. The primary colonization is of great importance since pioneer bacteria can modulate expression of genes in host epithelial cells, thus creating a favorable environment for themselves and inhibiting the growth of other bacteria introduced later into the ecosystem. The initial colonization in the newborn is therefore crucial for the composition of the permanent microbiota throughout life.

THE DIVERSITY OF THE INTESTINAL MICROBIOTA

Due to the presence of acid, bile pancreatic secretions and the phasic propulsive motor activity towards the ileum, a stable colonization of the stomach and duodenum is no easy feat for most microbes. As a result, the upper gastrointestinal tract hosts only few species of bacteria and harbors a low number of microorganisms, typically less than 10^3 bacteria per gram of content. The number of bacteria gradually increases along the jejunum and ileum, from approximately 10^4 in the jejunum up to 10^8 bacteria per gram of content at the terminal ileum[12]. Conversely, the colon harbors a complex and dense microbial ecosystem with bacterial counts up to 10^{12} living bacteria per gram of luminal content, which contributes to 60% of the fecal mass[13]. This ecosystem of microbiota consists of a core of native species that permanently colonize the lumen and a variable set of living bacteria that transit temporarily trough the tract.

The intestinal habitat of an adult individual contains 300-500 different species of bacteria of which the majority has never been cultivated and many are yet to be identified; however, only 30-40 species are accountable for 99% of the total population. Conventional bacteriological analysis of the fecal microbiota has shown that anaerobic bacteria outnumber aerobic microorganisms by a factor 100 to 1000. The dominant genera in the population of the adult human microbiota are *Bacteroides, Bifidobacterium, Eubacterium, Clostridium, Lactobacillus, Fusobacterium* and various Gram-positive cocci, whereas aerobes such as *Enterococcus* and *Enterobacteriaceae* are amongst the subdominant genera[14]. Every individual has a highly specific and unique combination of dominant and subdominant species, which is distinct from that found in

9 Fanaro *et al.*,2003
10 Yoshioka *et al.*,1983
11 Orrhage & Nord, 1999
12 O'Hara & Shanahan, 2006
13 Simon & Gorbach, 1984v
14 Guarner & Malagelada, 2003

other individuals and appears to be as personalized as a fingerprint. However, recent evidence indicates that fecal bacteria that can be cultivated outside of the gastrointestinal tract represent only a fraction of the bacteria actually present in the lumen. The use of culture-independent methods revealed that the diversity of the intestinal microbiota is considerably higher than anticipated so far[15]. Some data even suggest that each individual harbors unique strains[16].

PRIMARY FUNCTIONS OF INTESTINAL MICROBIOTA

A number of bacterial species present in the lumen can act as pathogens when the integrity of the mucosal barrier is impaired. Normally however, commensal microbes and their hosts enjoy a symbiotic relationship. The host provides a hospitable and nutrient rich environment and a flourishing and diverse intestinal microbiota exert numerous beneficial effects on the host's health. Comparative studies of germ-free and colonized animals have shown the influence of commensal microbes on the development and differentiation of the intestinal epithelium and gut immune function and showed a lower intestinal motor activity in gnotobiotic animals[17]. The main effects of the intestinal microbiota can be divided into three categories: trophic, metabolic, and protective functions.

Trophic function of the microbiota

The trophic functions of the microbiota have been of great scientific interest over the last decade. Comparisons of conventionally housed and germ-free animals have revealed that intestinal microbes can control the proliferation and differentiation of epithelial cells. For instance, villi of the small intestine are longer and crypts are shorter and contain fewer cells in germ-free animals[18]. In addition, bacteria play a pivotal role in the development of the immune system, in terms of gut-associated lymphoid tissue (GALT) development[19], mucosal immunity[20], and the induction of oral tolerance[21]. Immediately after exposure to commensal microorganisms, the number of intraepithelial lymphocytes greatly expands[22]; germinal centers with immunoglobulin producing cells rapidly arise in follicles[23] and in the lamina propria, and concentrations of immunoglobulin substantially increase in serum[24]. Studies in gnotobiotic animals also showed that commensal microbes modulate the fine-tuning of T-cell repertoires and T-helper (Th)-cell type-1 or type-2 cytokine profiles[25]. Thus, it is possible that the unique composition of the intestinal microbiota contributes to the variations in immune function between individuals.

15 Blaut *et al.*,2002
16 Kimura *et al.*,1997
17 Shanahan, 2002
18 Alam *et al.*,1994
19 Rhee *et al.*,2004
20 Williams *et al.*,2006
21 Noverr & Huffnagle, 2005
22 Helgeland *et al.*,1996
23 Weinstein & Cebra, 1991
24 Butler *et al.*,2000
25 Shanahan, 2002

Metabolic function of the microbiota

The metabolic function of intestinal microbiota consists of fermentation of non-digestible dietary residue and endogenous mucus produced by the epithelial layer. The total microbial population of an individual can be considered as a "microbe organ" – an organ similar to the liver in size (1-1.5 kg in weight) as well as in the number of biochemical transformations and metabolic reactions in which it participates[26]. With a combined microbial genome well in excess of the human genome, the intestinal microbiota provides various enzymes and biochemical pathways that are distinct from the host's own resources[27]. The resident bacteria in the colon represent an anaerobic bioreactor, serving in the breakdown of non-digested and otherwise indigestible polysaccharides[28]), the synthesis of micronutrients including vitamins[29], short chain fatty acids[30] and polyamines[31], and the absorption trace elements[32]. Since these complex metabolic activities result in recovery of metabolic energy and absorbable substrates for the host and supply of energy and nutritive products for bacterial proliferation this mutually beneficial metabolic relationship between bacteria and host has been referred to as commensalism[33]. Recent evidence underscored the importance of caloric extraction by microbiota. The microbiota of obese mice is rich in genes encoding enzymes that break down otherwise indigestible dietary polysaccharides, thereby influencing the efficiency of caloric extraction from food, which, in turn, may contribute to inter-individual differences in body weight[34].

Protective function of the microbiota

Commensal bacteria are known to provide an excellent hurdle against the colonization of the host by pathogenic microorganisms, a phenomenon known as "bacterial antagonism", or "colonization resistance". To inhibit the colonization by pathogens, commensal bacteria use assets such as production of antimicrobial substances (bacteriocins, microbiocidal compounds released by bacteria to kill competitor strains), pH modification of the luminal content, and competition for nutrients required for growth of pathogens[35]. Furthermore, the gene expression induced in the enterocytes by the initial colonizing microbiota provide the resident commensal microorganisms with a home field advantage, ensuring a stable micro ecosystem that resists colonization by transient pathogens. Moreover, various microorganisms (lactobacilli, bifidobacteria, and anaerobic bacteria) may occupy critical receptors on intestinal epithelial cells, which prevents pathogens from establishing a niche for persistence and proliferation[36]. Cross-talk between the microbiota and host epithelial cells further ensures that the host actively provides adequate amounts of specific nutrients required by the commensal microorganisms. This symbiotic relationship

26 Cummings & Macfarlane, 1997
27 Shanahan, 2002
28 Hooper *et al.*,2002
29 Hill, 1997
30 Schaible & Kaufmann, 2005
31 Noack *et al.*,1998
32 Schaible & Kaufmann, 2005
33 Commensalism is derived from the English word commensal, indicating 'the sharing of food' and originates from the Latin com mensa, meaning 'sharing a table'.
34 Turnbaugh *et al.*,2006; Ley *et al.*,2006
35 Lievin *et al.*,2000
36 Gordon, 2005

prevents unwanted overproduction of nutrients, which would favor proliferation of microbial competitors with potential pathogenicity to the host[37].

Commensal bacteria also directly interact with the intestinal epithelium via toll-like receptors (TLRs) on the epithelial surface. The family of toll-like receptors plays a crucial role in host-defense mechanisms by recognizing bacteria and their toxic products. Rakoff-Nahoum and colleagues (2004) found severe mortality and morbidity upon mucosal injury provoked by dextran sulfate sodium in mice deficient in MyD88, an adaptor molecule essential for TLR-mediated cell signaling. In other words, activation of toll-like receptors by commensal bacteria may maintain epithelial homeostasis by preventing mucosal injury and its associated complications. The gut microbiota should thus be included as a part of intestinal barrier function and an important integral component of the innate immune function of the gut.

DYSBIOSIS

The mucosa is constantly exposed to bacterial products that may exert deleterious effects on the epithelial layer and thus on host health. The presence of many of these toxic metabolites is a direct result of the type of fermentation that occurs in the lumen, which is dependent on the composition of the intestinal microbiota as well as substrates available for fermentation. For instance, high-protein diets contribute to production of potentially toxic products, which was referred to as bowel toxemia[38]. The history of the bowel toxemia theory is extending as far back as Hippocrates, who stated in 400 BC that, "death sits in the bowels" and "bad digestion is the root of all evil". In the late nineteenth century, Kuhne put forward that a predominantly vegetarian diet would prevent build up of intestinal toxins and hence would prevent disease[39]. The bowel toxemia theory eventually evolved into the intestinal dysbiosis theory.

Dysbiosis has been defined as a state, in which the microbiota exerts adverse effects by either qualitative and or quantitative changes in the microflora itself, or changes in the local distribution of commensal microorganisms or changes in their metabolic activity. These factors result in alterations in bacterial metabolism, as well as the overgrowth of potentially pathogenic microorganisms. Nevertheless, causing disturbances in the highly regulated and protected environment of a colonized intestinal tract is, as mentioned above, no easy feat for most pathogens because of the presence of acid, immunoglobulin (Ig)A, mucus, peristalsis, and the commensal microbiota themselves, that act in concert to resist colonization by transient pathogens. This is the reason why the prevailing ecological paradigm for the adult intestinal microbiota, now since years, is that each region of the intestine harbors a stable community of microorganisms[40]. Nevertheless, certain factors that can influence the composition of the intestinal microbiota have been defined, including dietary

37 Guarner & Malagelada, 2003
38 Cummings & Macfarlane, 1997
39 Hawrelak & Myers, 2004
40 Savage, 1977

changes[41], antimicrobial therapy[42], radiation[43], altered gut motility[44] and psychological[45] and physical stress[46].

Diet

Diet is an obvious potential niche-determining factor in the adult colon, especially given the fact that the intestinal microbiota of infants is influenced by transitions between breast-milk, formula feeding and solid foods[47]. Early studies, however, comparing broadly defined diets (e.g. 'Western' versus 'Asian') or manipulating the proportion of food categories, found only small effects involving few genera[48]. On the other hand, comparative studies with chemically well-defined diets have shown more pronounced effects. For instance, dietary sulphate favors sulphate-reducing bacteria over methanogenic bacteria[49], and inulin and related fibers stimulate the proliferation of bifidobacteria when this genus is rare[50]. Nevertheless the influence of periodic diet changes on the composition of the intestinal microbiota is probably not persistent at a phylogenetic level. The strains of many intestinal genera are comparable in growth abilities and have overlapping ranges of nutrient substrates[51]. Finally, mucus and other nutrients provided by the host are sufficient in supporting a diverse microbiota regardless of diet[52].

Antimicrobial therapy

Administration of antimicrobial agents causes disturbances in the ecological balance between host and microorganisms. To what extent disturbances occur depends on the spectrum of the agent, the dose, the route of administration, pharmacokinetic and pharmacodynamic properties, and *in vivo* inactivation of the agent. The effect of antimicrobial therapy on the intestinal microbiota and especially on its function as a barrier against colonization by potentially pathogenic bacteria can be devastating. Studies investigating anti-*Helicobacter pylori* agents have shown that the microbiota is significantly altered during treatment, most notably a substantial decrease in the numbers of lactobacilli and bifidobacteria[53]. Moreover, Myllyluoma and colleges (2007) showed decreased levels of *lactobacillus* and *enterococcus* species up to 70 days after a seven day antimicrobial treatment with lansoprazole, clarithromycin and amoxicillin. Furthermore, overgrowth of already present pathogenic microorganisms such as fungi or *Clostridium difficile*, is a frequent cause of antibiotic-associated diarrhea[54].

41 Kolida *et al.*,2002
42 Sullivan *et al.*,2001
43 Husebye *et al.*,1995
44 Barbara *et al.*,2005
45 Lizko, 1991
46 Alverdy *et al.*,2003
47 Favier *et al.*,2002
48 Finegold & Sutter, 1978
49 Gibson *et al.*,1993
50 Kolida *et al.*,2002
51 Gibson *et al.*,1993; Flint, 2004
52 Corfield *et al.*,1992
53 Buhling *et al.*,2001
54 Hurley & Nguyen, 2002

THE EFFECTS OF PSYCHOLOGICAL STRESS ON THE INTESTINAL MICROBIOTA

It is increasingly recognized that various types of stress have a major impact on intestinal physiology, and may thereby cause intestinal dysfunction and/or diseases. For example, it has been established that early life stress and sustained stressful life events predispose humans to the development of functional bowel conditions, such as irritable bowel syndrome (IBS)[55]. In addition, stress seems to affect chronic inflammatory bowel diseases (IBD)[56]. Our understanding of stress-induced brain-gut-microbiota interactions and their effects on intestinal functions has progressed during recent years.

EFFECTS OF STRESS ON INTESTINAL FUNCTION

One of the mechanisms connecting psychological stress and gastrointestinal diseases is stress-induced effects on mucosal barrier function. Small quantities of luminal antigens cross the epithelium and interact with the immune system as an important surveillance of the luminal contents. The ability to control this uptake is denoted; *intestinal barrier function*. Excessive uptake of antigens and bacteria, for example during stress, may over-activate the immune system and lead to local mucosal inflammation or even sepsis, when bacteria and toxins are translocated into the systemic circulation.

Stress also affects gastrointestinal motility. Almy and Cannon clearly demonstrated in the first half of the last century that emotional stress has a profound impact on gastric and colonic motor activity in man and cats[57]. In both human and animal experimental studies it was shown that acute stressors could delay gastric emptying, increase small bowel transit and increase colonic motility. Recently the behavioral, endocrine, immune, autonomic, and visceral adaptive stress responses have been reviewed by Yvette Taché and Bruno Bonaz (2007) and David Grundy *et al*. (2006). Over the past few decades, corticotrophin-releasing hormone (CRH) signaling pathways have been shown to be the main coordinators of the endocrine, behavioral, and immune responses to stress. Emerging evidence links the activation of CRH receptors type-1 and type-2 with stress–related alterations of gut motor function and regulation of barrier function[58]. Chronic psychological stress models, such as 1 hour per day water avoidance and neonatal maternal separation, also show a modulation of autonomic nervous system outflow, an activation of the hypothalamic-pituitary-adrenal axis and alterations in pain modulatory mechanisms. These mechanisms can be associated with changes in gastrointestinal motility[59] and visceral sensitivity[60], and

55 Mayer *et al.*,2000
56 Collins *et al.*,2001
57 Almy *et al.*,1949; Cannon, 1953
58 Wallon *et al.*,2007
59 Tache & Perdue, 2004
60 Barreau *et al.*,2004; Stam *et al.*,1997

mucosal function[61]. The disturbing influence on gastrointestinal motility and barrier function by chronic psychological stress may also affect the intestinal microbiota, inducing bacterial overgrowth with anaerobic and gram-negative bacteria in the small bowel and a decrease in lactobacilli and *Bifidobacterium* concentrations.

STRESS AND COMPOSITION OF MICROBIOTA

Several lines of evidence suggest that psychological stress may have important effects on the intestinal microbiota in animals and humans. Under normal conditions, the microenvironment of the mammalian intestinal epithelium is relatively sterile[62]. However, under conditions of stress and dietary manipulation, an increased number of gram-negative bacteria can be found in dense association with the intestinal mucosal epithelium[63]. To determine whether psychological stress results in an altered composition of microbiota, Bailey and Coe (1999) investigated changes in endogenous microbiota in primates after maternal separation. The stability of the indigenous microbiota was evaluated in fecal samples taken from 20 infant rhesus monkeys before and up to seven days after maternal separation. A significant decrease in fecal bacteria, especially lactoba*cilli*, was evident on day three postseparation, with a return to baseline levels by the end of the week, suggesting that psychological stress can alter the composition of intestinal microbiota for several days. In addition, evidence suggests that lactobaci*lli* respond to stress-induced changes in the intestinal physiology, such as inhibition of gastric acid release[64], alterations in gastrointestinal motility, or increased duodenal bicarbonate production[65]. These changes may result in an intestinal environment less conducive to lactobacilli survival, adherence, and proliferation. The effects of psychological stress on the human intestinal microbiota were studied in Soviet cosmonauts. Lizko and colleagues (1991) revealed that during the preparation phase, immediately before take-off, and after landing the microbiota of cosmonauts showed a distinct decrease in fecal bifidobacteria and lactobacilli counts as well as a substantial increase in the numbers of *Escherichia coli* due to the emotional stress. This also led to a subsequent decline in colonization resistance, which in turn resulted in increased numbers of potentially pathogenic organisms.

Catecholamines and microbiota

Exposure to stress has been documented to result in a dramatic increase in catecholamine levels, and mesenteric organs are thought to contribute substantially to the total body production of norepinephrine (~50% of the norepinephrine formed in the body under normal conditions)[66]. These high concentrations of norepinephrine also spill over into the lumen of the gastrointestinal tract and evidence shows that catecholamines have a profound effects on intestinal microbiota. Holdeman *et al.* (1976) studied stress-induced factors that affect the human fecal flora. They noted a 20-30 percent rise in the proportion of *Bacteroides fragilis* subsp. *thetaiotaomicron* in the feaces of individuals in response to anger or fearful situations. When

61 Gareau *et al.*,2007
62 Amann *et al.*,1990
63 Martin *et al.*,2004
64 Lenz & Druge, 1990
65 Lenz, 1989
66 Eisenhofer *et al.*,1995

these situations were resolved, the concentration of these organisms in the feaces decreased to normal levels. This effect was thought to be mediated via epinephrine, which has been shown to stimulate both intestinal motility and bile flow, and growth of *B. fragilis* subsp. *thetaiotaomicron* is enhanced by bile[67]. *In vitro* experiments conducted by Lyte *et al.* (1992) demonstrated that several catecholamines have the ability to enhance the growth of *E. coli*, *Yersinia enterocolitica* and *Pseudomonas aeruginosa*. Among the various catecholamines tested, norepinephrine consistently induced the greatest increase in growth of all strains. Moreover, it was demonstrated that upon exposure to norepinephrine, *E. coli* produces a growth hormone known as an "autoinducer of growth", stimulating growth of many gram-negative bacteria[68]. These growth-promoting effects on *E. coli* were also confirmed *in vivo* in a murine model of trauma-induced norepinephrine release[69].

Mucin, IgA and microbiota in stress

Chronic psychological stress in animal models results in a significant reduction in the production of mucin, by mechanisms involving catecholamines and mast cells, and leads to a decreased presence of acidic mucopolysaccharides on the mucosal surface[70]. Since both mucin and acidic mucopolysaccharides are important for inhibiting adherence of pathogenic organisms to the gut mucosa, a decrease in either would contribute significantly to increased colonization by pathogenic organisms. Consequently, the stress-induced decrease in mucus is associated with a higher number of adhering bacteria[71], and enhanced susceptibility to hapten-induced colitis[72].

As IgA plays a vital role in the defense against pathogenic organisms by inhibiting bacterial adherence and promoting their elimination from the intestinal lumen, any decrease in IgA secretion would most likely decrease colonization resistance against potentially pathogenic microorganisms[73]. Mimicking stress with a high dose dexamethasone in rodents resulted in impaired colonic IgA-secretion together with increased permeability and enhanced bacterial adherence to the mucosa[74]. Moreover, studies on college students showed decreased secretory IgA concentrations during or shortly after examinations[75]. In children, Drummond *et al.* (1997) found that stressful life events lead to lower levels of secretory IgA. Salivary concentrations of IgA showed an inverse correlation with norepinephrine concentrations, suggesting that sympathetic nervous system activation suppresses the production and/or release of IgA[76]. Enteric nerves and norepinephrine may also be involved in regulation of bacterial uptake. Transmucosal transport of *E. coli* K-12 across follicle-associated epithelium was increased 30-fold by chronic stress in rats[77], and mechanisms of epithelial internalization of bacteria in follicle-associated epithelium seem to involve norepinephrine and enteric nerves[78]. Frequent suppression of intestinal barrier

67 Moore *et al.*,1978
68 Freestone *et al.*,1999
69 Lyte & Bailey, 1997
70 Lizko, 1987
71 Soderholm *et al.*,2002
72 Qiu *et al.*,1999
73 Lizko, 1987
74 Spitz *et al.*,1996
75 Jemmott, III & Magloire, 1988
76 Drummond & Hewson-Bower, 1997
77 Velin *et al.*,2004
78 Green, 2003

function and mucosal immunity by the sympathetic nervous system during stressful experiences could therefore contribute to colonization of the intestinal mucosa by potential pathogens.

The effects of severe stress and critical illness on the intestinal microbiota

A large body of evidence has shown that stress is involved in the pathogenesis of gastric stress ulcers in critically ill patients[79], as well as in peptic ulcer disease[80]. Mechanisms include changes in the prostaglandin-regulated secretion of mucus and the obstruction of back-diffusion of acid in the normally very firm tight junctions of the gastric surface epithelium[81]. Moreover, a number of studies have documented intestinal pathophysiology in critically ill patients, e.g. following trauma, sepsis, or after major surgery[82]. Translocation of gut bacteria and toxins to lymph nodes and/or the systemic circulation has also been found in these patients[83]. It was also shown that the degree of barrier dysfunction in patients admitted to intensive care units was related to the development of multiple organ dysfunction syndrome[84]. This relation could, however, not be reproduced in less ill patients undergoing major upper gastrointestinal surgery[85]. There are a number of possible mechanisms of mucosal barrier dysfunction in these patients. For example, mucosal hypoxia and acidosis due to hypoperfusion, leads to oxidative stress and adenosine triphosphate (ATP) depletion during reperfusion, which may induce increased levels of pro-inflammatory cytokines[86]. Another possible mechanism is the extensive focal apoptosis of crypt epithelial cells and lamina propria lymphocytes found in the intestine of patients with severe trauma[87]. Increased crypt cell apoptosis has also been described in the small bowel of rats subjected to chronic stress[88] as well as in sepsis models in mice[89]. In addition, the importance of the neuroendocrine stress response in patients following surgical trauma is shown by the positive clinical effects of epidural analgesia in the postoperative course after major gastro-intestinal surgery[90]. Epidural analgesia, which blocks the regional innervation to the intestine and interrupts the transmission of pain from the area, attenuates the stress response, as shown by lower postoperative levels of adrenocorticotropic hormone (ACTH), cortisol, aldosterone and glucose[91].

Selective use of virulence genes

Bacteria can sense their environment, count their population density, activate virulence genes, and achieve mobility in social groups by sophisticated communication systems of which the precise molecular dialogue is just beginning to be elucidated. Merz *et al.* (2000) conducted an elegant study where they were able to demonstrate these systems, showing that bacteria gather information and create their

79 Cook *et al.*,1996
80 Levenstein *et al.*,1999
81 Werther, 2000
82 Aranow & Fink, 1996
83 Sedman *et al.*,1994
84 Doig *et al.*,1998
85 Kanwar *et al.*,2000
86 Aranow & Fink, 1996
87 Hotchkiss *et al.*,2000
88 Boudry *et al.*,2007
89 Coopersmith *et al.*,2002
90 Fotiadis *et al.*,2004
91 Kouraklis *et al.*,2000

own communication networks within colonies. For example, when separating a single bacterium from its colony by microforceps, it becomes retractile and twitches to move toward its group, suggesting ability to sense position and remoteness from the group. In addition, bacteria may also sense crowding conditions and subsequently reduce proliferation to avoid outgrowing their resources[92]. One such system by which bacteria communicate in a cell-density-dependent manner is referred to as *quorum sensing signaling system*, which regulates, for instance, the *P. aeruginosa* virulence factor PA-1[93]. The logic behind this system is to enable bacteria to sense their population density and express virulence genes only when a critical mass is present, presumably that amount needed to guarantee successful opportunism against the host. As bacteria gain information through this chemical communication system, they can launch a surprise attack on the host, employing an array of virulence factors in a coordinated and overwhelming manner.

Alverdy *et al.* (2003) has put forward that gut-derived sepsis occurs when the right pathogen with the right virulence factors meets the right host, i.e. it results from disrupted host-pathogen interactions that take place within the intestine during severe stress. The sensory input systems, e.g. quorum sensing, allow pathogens to detect changes in their local environment and to assess costs versus benefits of the activation of virulence genes, because bacteria cannot afford to have their virulence gene products constantly expressed when they are not needed. For example, when competition for nutrients is low as in laboratory conditions with nutrient-rich growth media, virulence genes are switched off and energy resources conserved[94]. Yet, changes in the local milieu, such as changes in pH, nutrient availability, osmolality, redox state or adaptive elements of the immune system such as interferon-γ [95], tumor necrosis factor (TNF)-α[96], and interleukin (IL)-1[97], as well as innate elements including adenosine[98], epinephrine[99], and antimicrobial peptides[100], can induce the expression of virulence gene products at any given moment. For instance, certain strains of the human opportunistic pathogen *P. aeruginosa* are, in high concentrations, lethal to *Caenorhabditis elegans*. By simply changing the osmolality of the agar on which the *C. elegans* is grown, the killing effect of *P. aeruginosa* on these worms is enhanced[101]. Similar experiments have been performed altering oxygen concentration. For example, *Salmonella* only induce morphologic and cytoskeletal changes in cultured epithelial cells, correlating with an epithelial barrier dysfunction, when grown under low-oxygen conditions[102]. Furthermore, in this regard, *P. aeruginosa* is able to activate its virulence circuitry in response to soluble elements of epithelial hypoxia and reoxygenation. Using the human intestinal epithelial cell line Caco-2, Kohler *et al.* (2005) demonstrated that soluble compounds released by hypoxic and/or reoxygenated cultured intestinal epithelial cells, induced the expression of a key virulence protein in *P. aeruginosa*,

92 Schiemann & Olson, 1984
93 Winzer *et al.*,2000
94 Foster & Spector, 1995
95 Wu *et al.*,2005
96 Luo *et al.*,1993
97 Porat *et al.*,1991
98 Kohler *et al.*,2005
99 Sperandio *et al.*,2003; Alverdy *et al.*,2000
100 Bader *et al.*,2005; Hancock & McPhee, 2005
101 Tan *et al.*,1999
102 Francis *et al.*,1992

PA-I lectin/adhesin. It was previously shown by Laughlin *et al.* (2000) that PA-I lectin of *P. aeruginosa* induces a defect in intestinal barrier function that allows exotoxin A to cross the epithelium, resulting in lethal gut-derived sepsis in mice. It is shown that under hypoxic conditions a symbiotic host-pathogen relation can erode into an escalating arms race. Evidence shows that during intestinal epithelial hypoxia, eukaryotic cells activate a cytoprotective barrier-enhancing response to invading pathogens in association with hypoxia-inducible factor (HIF)-1α expression and extracellular adenosine release. However, under hypoxic conditions in *P. aeruginosa*, not only induction of the potent barrier deregulating protein PA-I lectin occurs[103], also the enzyme of *P. aeruginosa* that metabolize adenosine (adenosine deaminase) is upregulated in response to medium from hypoxic epithelial cells, suggesting that *P. aeruginosa* may have evolved a very clever virulence tactic to deplete epithelial cells of a major cytoprotective compound, rendering them all the more vulnerable to the effects of its expressed virulence genes[104]. Moreover, the discovery that norepinephrine can induce bacterial virulence gene expression in *E. coli*, resulting in increased adherence to murine cecal mucosa in Ussing chambers[105], provides additional evidence that potential pathogens recognize host stress and react with enhanced virulence.

Osmolality, pH, oxygen concentration, nutrient availability, and norepinephrine concentrations can be easily imagined to be altered in the intestinal tract of a critically ill patient on vasoactive pressors, artificial nutrition, and powerful sedatives and opiates. Therefore, a balance between incoming signals and available resources is likely to dictate whether a microbe remains an indolent colonizer or switches its phenotype to that of a life-threatening pathogen in critically ill patients. Animal studies support the hypothesis that the same environmental cues seem to play a role in the context of surgical stress. For example, Wu *et al.* (2005) showed that in mice subjected to surgical stress (30% surgical hepatectomy), the expression of the barrier-disrupting virulence factor, PA-I lectin/adhesin was increased, in luminal *P. aeruginosa*. In addition, hepatectomy and starvation in mice shifts the composition of intestinal *E. coli* to that of a more adherent strain capable of altering the permeability of cultured mouse colon cells[106]. Furthermore, Alverdy *et al.* (1999) have shown a 20-fold increase in numbers of luminal *E. coli* as well as an increase in mucosa-associated *E. coli* in cecum of mice after hepatectomy and short-term starvation.

There is a growing body of evidence suggesting that stressed hosts perceive not only pathogens, but also commensals as a threat. Using monolayers of human colonic epithelial cells Nazli *et al.* (2004) showed that only in chemically stressed tissues and not in controls, commensal bacteria initiate nuclear factor-κB (NF-κB) signaling, production of IL-8 and cause barrier dysfunction. These data suggest that under metabolic stress, the intestinal epithelium perceives a normally harmless bacterium as a threat, resulting in loss of barrier integrity, augmented penetration of bacteria into the mucosa, and upregulation of chemokines. In additional experiments it was shown that non-pathogenic *E. coli* reduce the expression of tight junction proteins and alter the cytoskeleton in enteric epithelia under metabolic stress[107]

103 Kohler *et al.*,2005
104 Patel *et al.*,2007
105 Chen *et al.*,2003
106 Rocha et al.,2001
107 Nazli *et al.*,2006

PROBIOTICS

HISTORY AND DEFINITION

There is currently a growing appreciation for the potential of commensal organisms to influence host health. The market for probiotics has considerably grown in recent years together with an increasing number of probiotic-containing commercial products claiming specific health benefits. Probiotic food products represent a considerable part of the functional food market, and continues to grow at an exponential rate, with the potential for market growth estimated at an astounding US$120 million per month[108]. The therapeutic use of food fermented with microorganisms was already described in the Persian version of the Old Testament, stating that "Abraham owed his longevity to the consumption of sour milk". In classical Roman literature, as early as 78 BC, gastroenteritis was recommended to be treated by ingestion of fermented milk products. However, the term probiotics was only coined in 1965 by Lilly and Stillwell (1965) to describe a growth-promoting effect of the ciliate *Tetrahymena* by a factor secreted by protozoan microorganisms. The current definition of the Joint Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization states that: "Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host"[109].

The Nobel Prize laureate Elie Metchnikoff was the first to scientifically investigate this ancient concept of health beneficial fermented milk products and described in 1907 that the lactic acid producing strain *Lactobacillus bulgaricus* contained in yoghurt is able to displace pathological intestinal flora. In 1917 in the pre-antibiotic era, Alfred Nissle isolated the Nissle *E. coli* strain from the stool of a soldier during World War I who remained healthy, despite the fact that most of his comrades were combating diarrhea. This was done, with the therapeutic purpose to treat a 20 year old female suffering from active ulcerative colitis. Despite the fact that remission was achieved after 5 weeks of treatment[110], the concept of therapeutic use of probiotics disappeared into the academic backroom and was ridiculed until the major advances in the scientific field were made. At present there is a rapidly growing body of evidence to support the concept of therapeutic use of probiotics. To underline the current interest in the scientific field of probiotics related to human health, roughly 500 peer reviewed papers and 180 literature reviews were published with this key phrase in the course of the last year (2006).

DIFFERENT PROBIOTIC STRAINS

In literature, various health promoting effects have been attributed to a variety of bacteria. The range of potential probiotic species can be categorized into two distinct groups: lactic acid bacteria and other probiotic species.

108 Senok *et al.*,2005
109 Pineiro & Stanton, 2007
110 Nissle, 1918

Lactic Acid Bacteria

The first group includes Lactic Acid Bacteria (LAB), which have been used in fermented milk and yoghurt throughout the history of mankind. LAB represent a group of bacteria that are functionally related by their ability to produce lactic acid during fermentation. The acidification and enzymatic processes associated with the growth of LAB impart the key flavor and consistency to a variety of fermented foods, predominantly milk and dietary products, as well as preservative qualities. Bacteriocins are produced by several LAB strains and provide an additional hurdle for growth of pathogenic microorganisms and are thereby contributing to these preservative qualities. Most of the industrial applications of LAB rely on 7 key beneficial and non pathogenic species: *Bifidobacterium* (occurs in dietary products), *Lactococcus* (present in fermented milk products), *Lactobacillus* (applied in milk, meat, vegetables and cereal), *Leuconostoc* (applied in vegetables and milk), *Pediococcus* (applied in vegetables, meat and milk), *Oenococcus oeni* (employed in wineries) and *Streptococcus thermophilus* (employed as a starter culture for the manufacture of several important fermented dairy foods, including yogurt and Mozzarella cheese). Because of their practical implications in fermentation, bio-processing, agriculture, food, and, more recently, medicine, LAB have been the focus of extensive genomic sequencing efforts, with currently at least 6 completed genomes[111].

The most commonly used LAB-members are bifidobacteria and lactobacilli. Bifidobacteria are capable of synthesizing vitamins (mainly B vitamins) and digestive enzymes such as casein phosphatase and lysozyme[112]. Six of the 38 known species of bifidobacteria are frequently used as probiotics and have been shown to be beneficial in a wide range of applications, including attenuation of infectious diarrhea[113], protection against colonizing pathogens[114], stimulation of the immune system[115] and possibly protection of the host from carcinogenic activity by the intestinal microbiota[116]. Another important group of probiotic bacteria constitute of lactobacilli. Lactobacilli produce hydrogen peroxide[117], lower intestinal pH[118] and synthesize bacteriocins which inhibit growth of pathogenic bacteria[119].

Other probiotic species

The second group of allegedly probiotic bacteria consists of genera which are normally not regarded as safe. The principle 'fight bacterial pathogens with their non-pathogenic relatives' has raised the idea of applying non-pathogenic isolates, such as certain *E. coli* strains, as probiotics. There is evidence showing that various *E. coli* strains reduce the incidence of diarrhea[120] and allergies[121], maintain remission in inflammatory bowel diseases[122] and prevent colonization with nosocomial

111 Klaenhammer *et al.*,2007
112 Gibson *et al.*,1995
113 Maier & Hentges, 1972
114 Corr *et al.*,2007
115 Klein *et al.*,2007
116 Rafter *et al.*,2007
117 Naaber *et al.*,2004
118 Corr *et al.*,2007
119 Klaenhammer *et al.*,2007
120 Henker *et al.*,2007
121 Hawrylowicz & O'Garra, 2005
122 Kruis *et al.*,2004

pathogens in preterm infants[123]. *Clostridium butryricum* has also been suggested to be a probiotic strain due to its ability to produce butyric acid. This short chain fatty acid is an important energy source for the colonic epithelium (providing 60-70% of its energy requirement) and is suggested to have anti-inflammatory activity[124]. And indeed, treatment with *C. butryricum* of IBD[125] as well as antibiotic-associated diarrhea in children[126] shows great promise. Another potential probiotic is *Saccharomyces boulardii,* a tropical strain of yeast which was first isolated from lychee and mangosteen fruit in 1923 by French scientist Henri Boulard. Due to its natural resistance to antibiotics and gastric acidity, *S. boulardii* is a candidate probiotic to treat antibiotic-associated diarrhea[127]. Its effectiveness may largely rely on its potential to produce a specific protease alleviating the intestinal effects of *Clostridium difficile* toxin A[128]. Although it has also been shown to increase the local production of secretory IgA [129] and to decrease the inflammatory process, especially through the inhibition of the NF-κB translocation into the nucleus[130].

123 Lodinova-Zadnikova & Sonnenborn, 1997
124 Malago *et al.*,2005
125 Araki *et al.*,2004
126 Seki *et al.*,2003
127 Szajewska & Mrukowicz, 2005
128 Pothoulakis *et al.*,1993
129 Buts *et al.*,1990
130 Sougioultzis *et al.*,2006

PROBIOTICS AND STRESS

EFFECTS OF PROBIOTICS IN STRESS-INDUCED PATHOPHYSIOLOGY

In clinical studies there is indirect evidence of probiotics counteracting the consequences of stress. A number of randomized trials have shown positive effects of probiotics in infectious enteritides with disturbances in intestinal barrier function, e.g. *C. difficile*-induced diarrhea[131]. Moreover, probiotics have been shown to have clear positive effects in maintaining remission in pouchitis[132] and ulcerative colitis[133]. There are also a number of randomized controlled trials showing that probiotics can reduce the colonization of the upper gastrointestinal tract[134] and prevent secondary infections in patients with surgical trauma or surgical infections[135]. Most studies are however too small to show conclusive data, and the results from different studies are conflicting.

Using animal models, and thereby gaining reproducible and coherent study groups, it has been much easier to show beneficial effects of probiotics in intestinal disease models and trauma models, as well as in various stress models. For example, the multistrain probiotic mixture, VSL#3, normalizes epithelial function and diminishes colitis in IL-$10^{-/-}$ mice[136]. Studying the effects of probiotics on gut dysfunction in two different models of postinfective irritable bowel syndrome in mice, Verdu *et al.* (2006) showed that *Lactobacillus paracasei* normalized muscle hypercontractility. In an ongoing project we have found that another multistrain probiotic, Ecologic® 641, prevents ileal barrier dysfunction and restores tight junction protein expression in an acute pancreatitis model[137].

In more traditional animal models of chronic psychological stress, a number of lactic acid bacteria have shown the ability to counteract stress-induced intestinal pathophysiology. In a rat model of chronic stress with daily water avoidance stress, Zareie *et al.* (2006) found that the combination of *Lactobacillus helveticus* and *Lactobacillus rhamnosus* in the drinking water normalized small bowel ion secretion and prevented bacterial translocation to lymph nodes by inhibiting adhesion to the epithelial cells. Rat pups stressed by maternal separation develop persistent mucosal barrier dysfunction, including an impaired host defense to luminal bacteria[138]. Using the same probiotic strains as in Zareie's study, Gareau *et al.* (2007) showed that probiotic treatment twice daily during the separation period, normalized colonic ion transport, macromolecular permeability and adherence of commensal bacterial to the epithelial surface. Moreover, these beneficial effects were maintained until adulthood, diminishing the increased stress sensitivity found in adult rats exposed to maternal separation during the first weeks of life. These findings are in line with results of Eutamene and co-workers (2007), studying intestinal ^{51}Cr-EDTA permeability and visceral sensitivity in a similar maternal separation model as well as in

131 Johnston *et al.*,2007
132 Gionchetti *et al.*,2000
133 Kruis *et al.*,2004
134 Gotteland *et al.*,2005; Anderson *et al.*,2004; McNaught *et al.*,2002
135 Nomura *et al.*,2007
136 Madsen *et al.*,1999
137 Lutgendorff *et al.*,2009
138 Gareau *et al.*,2006

a partial restraint stress model in rats. It was found that *L. paracasei* prevented the stress-induced increase in permeability and sensitivity to colorectal distension. An important observation in this study was that the positive effects were strain specific; the protective effects of *L. paracasei* were not reproduced by *Lactobacillus johnsonii* or *Bifidobacterium lactis*. Similar findings were previously shown by Luyer *et al.* (2005) in a model of hemorrhagic shock in rats. Pretreatment for 7 days with *L. rhamnosus* prevented tight junction protein disruption and endotoxemia during hemorrhagic shock, whereas *Lactobacillus fermentum* had no substantial effect on shock-induced intestinal damage. These latter findings underline the importance of strain-specificity.

MECHANISMS OF PROBIOTIC ACTIONS

Probiotics have a number of functions and effects on intestinal physiology that could counteract the harmful consequences of stress. Several of these are directly or indirectly related to intestinal barrier function. The complex relationship of the indigenous microbiota with the host can be depicted in a multilevel framework, where luminal bacteria, the mucus layer, epithelial cells, innate and adaptive immunity, the enteric nervous system and intestinal motor activity all work in concert to maintain this unique symbiotic homeostasis (fig.1). The mechanisms by which probiotics sort their effect are highly complex and largely unknown, but can in broad terms be divided into interactions with each of these levels.

Luminal effects of probiotics

In the lumen, it was recently shown that inhibitory quorum sensing signals released from *Lactobacillus acidophilus* may reduce the transcription of genes important for intestinal colonization of *E. coli* O157:H7[139]. Moreover, *L. acidophilus* and *L. rhamnosus* as well as *S. thermophilus* have the ability to reduce the adhesion of pathogenic *E. coli* to human colonic cell lines[140]. Finally, some probiotics may interact with intestinal pathogens by competition for essential nutrients, modification of ecological conditions (pH, for instance), and competition for adhesion sites in the intestine.

Effects of probiotics on mucin secretion

Modulation of mucin secretion is another asset of the broad range of actions of probiotics to protect the mucosal barrier. Caballero-Franco and colleagues (2007) showed that orally administered VSL#3 increased the basal luminal mucin content by 60% and showed an upregulation of the mucin gene MUC-2 in healthy rats. Furthermore, *L. rhamnosus* GG increased mRNA expression and extracellular secretion of MUC3 mucin and inhibited adherence of enteropathogenic *E. coli* to HT29 intestinal epithelial cells, but not to non epithelial cells, suggesting a protective role of probiotics via mucin secretion[141].

Probiotic effects on epithelial cells

Probiotic strengthening of the mucosal barrier is another asset of their variety of beneficial functions. The probiotic strain *E. coli* Nissle 1917, but not other *E. coli*

139 Medellin-Pena *et al.*,2007
140 Resta-Lenert & Barrett, 2003
141 Mack *et al.*,2003

strains, strongly induces expression of antimicrobial peptide human beta-defensin-2 in Caco-2 intestinal epithelial cells and thereby stimulates the intestinal innate defense[142]. Activation of beta-defensin-2 can inhibit microbial growth of potential pathogens in the gastrointestinal tract and may therefore lead to the enhancement of the intestinal mucosal barrier. Furthermore, specific heat shock proteins (Hsps) known for their ability to maintain cytoskeletal integrity, were produced in intestinal epithelial cells exposed to VLS#3 conditioned medium[143]. In additional experiments it was shown that soluble factors of *L. rhamnosus* GG induce the same Hsps in mice colonic epithelial cells through activation of several mitogen-activated protein kinases (MAPKs)[144]. Similarly, *S. boulardii* abrogated the alterations induced by an enteropathogenic *E. coli* strain on transepithelial resistance and zonula occludens (ZO)-1 distribution in T84 cells, suggesting a protection of tight junctional integrity against the deleterious effects of *E. coli* infection[145]. This yeast and its culture supernatant were also found to prevent chloride secretion induced by *E. coli* toxin and cholera toxin. Moreover, the probiotic combinations VSL#3[146] and Ecologic® 641[147] have been shown to prevent increased intestinal permeability to macromolecules in colitis and acute pancreatitis, respectively, by mechanisms involving stabilization of the cytoskeleton and tight junction proteins.

Probiotics are also believed to function via the modulation of epithelial cell proliferation and apoptosis. The administration of 10^7 colony forming units (CFU)/ml of *L. rhamnosus* GG and *C. butyricum* in rats was shown to enhance epithelial cell proliferation rates in the small intestine, cecum and distal colon. This proliferation promoting effect is believed to be due to the capacity of probiotics to produce short fatty acids by fermentation of polysaccharides[148]. Anti-apoptotic effects of probiotics have been shown in an *in vitro* model, in which *L. rhamnosus* GG was able to prevent apoptosis in mouse and human colon cells through activation of the anti-apoptotic Akt/protein kinase B pathway and also via inhibition of the pro-apoptotic p38/MAPK pathway[149]. Subsequent experiments have shown that also soluble proteins produced by *L. rhamnosus* GG activated the Akt/protein kinase B pathway, inhibited cytokine-induced epithelial cell apoptosis, and promoted cell growth in human and mouse colon epithelial cells[150].

Immunomodulatory effects of probiotics

Amongst the numerous health benefits attributed to probiotic bacteria, their capacity to interact with the immune system of the host is one of the most extensively studied effects and is now supported by an increasing number of *in vitro* and *in vivo* experiments. Recognition of microorganisms by epithelial cells is based upon their detection of signature molecules, called microbe-associated molecular patterns (MAMP), by pattern recognition receptors in particular membrane TLRs. TLRs function to alert the host to the presence of microbes and are critical for the

142 Wehkamp *et al.*,2004
143 Petrof *et al.*,2004
144 Tao *et al.*,2006
145 Czerucka *et al.*,2000
146 Madsen *et al.*,2001
147 Lutgendorff *et al.*,2009
148 Ichikawa *et al.*,1999
149 Yan & Polk, 2002
150 Yan *et al.*,2007

initiation of inflammatory and immune defense responses. For example, TLR-2 recognizes lipoteichoic acid and bacterial lipoproteins abundant in the cell wall of gram-positive and gram-negative bacteria, TLR-4 recognizes lipopolysaccharide from gram-negative bacteria and TLR-9 recognizes bacterial DNA. Recently, Grabig and colleagues (2006) showed the positive effects of *E. coli* Nissle 1917 on dextran sodium sulfate-induced experimental colitis and decreased pro-inflammatory cytokine secretion in wild-type mice, but not in TLR-2 or TLR-4 knockout mice, indicating that *E. coli* Nissle 1917 ameliorates experimental induced colitis in mice via TLR-2- and TLR-4-dependent pathways. Interestingly, the positive effect of the probiotic mixture VSL#3 in the same model of colitis was also observed after administration of its extracted DNA and suppressed in TLR-9 deficient animals, showing that at least some of the immunomodulatory effects may be due to bacterial DNA and its recognition by TLR-9[151].

The NF-κB signaling pathway plays a central role in the host's immune response and is known to be influenced by host-microbe interactions. For example, it was via the NF-κB signaling pathway that the presence of *L. rhamnosus* GG reduced TNF-α-induced IL-8 production in Caco-2 intestinal epithelial cells[152]. Another mechanism was shown for VSL#3 in which the soluble factors produced by the probiotic mixture inhibit the degradation of the NF-κB inhibitor in mouse colonic epithelial cells by proteasome inhibition[153].

In addition, some probiotics are thought to stimulate the production of secretory IgA. For instance, Park and colleagues (2002) showed that *Bifidobacterium bifidum* significantly induced total IgA and IgM synthesis by both mesenteric lymph nodes and Peyer's patch cells. Since IgA secretion is an important immunological defense barrier to toxins and pathogens, IgA stimulation by *B. bifidum* may explain its effectiveness in several pathological conditions. Moreover, *Lactobacillus*-species may act via inhibition of interferon γ-induced barrier dysfunction[154], and by reducing the number of TNF-expressing lymphocytes in intestinal inflammation (ileal Crohn's disease)[155], thus having effects also on subepithelial immune cells.

Effects of probiotics on the enteric nervous system and motility

There is a small but growing body of evidence that probiotics exert effects on the enteric nervous system. It was shown in a rat model, that visceral pain, induced by colorectal distension, was prevented by treatment with *Lactobacillus reuteri,* showing an effect of *L. reuteri* on enteric nerves[156]. In line with these results was previously shown in the same colorectal mouse model while antibiotic treatment induced visceral sensitivity, that administration of *L. paracasei* in spent culture medium normalized visceral sensitivity, indicating that perturbations in gut flora and in inflammatory cell activity alter sensory neurotransmitter content in the colon, and result in altered visceral perception[157]. Moreover, Rosseaux *et al.* (2007) recently showed in an intriguing study that *L. acidophilus* NCFM induced a strain-specific reduction of

151 Rachmilewitz *et al.*,2004
152 Zhang *et al.*,2005
153 Petrof *et al.*,2004
154 Resta-Lenert & Barrett, 2006
155 Borruel *et al.*,2002
156 Kamiya *et al.*,2006
157 Verdu *et al.*,2006

intestinal pain perception mediated via cannabinoid and m-opioid receptors. There is also indirect evidence from several clinical studies in IBS patients suggesting that probiotic strains affect the enteric nervous system[158].

Another aspect is that the endogenous flora influences transit through the gastrointestinal tract. A randomized controlled trial showed that *Bifidobacterium animalis* DN-173010 shortened the transit time in sigmoid colon in healthy women[159]. This probiotic did not affect fecal weight, pH, bacterial mass, or the fecal bile acids and the mechanism of its effect on colonic transit remains unknown.

158 Shanahan, 2007; Spiller, 2007
159 Marteau *et al.*,2002

PROBIOTICS – CURRENT ISSUES AND FUTURE PERSPECTIVES

SAFETY OF PROBIOTIC STRAINS

Although most probiotic strains have a long history of safe use in practice, and LAB have a "Generally Regarded As Safe (GRAS)" status due to their ubiquitous appearance in food, probiotics may in theory be responsible for four types of adverse effects: infections, deleterious metabolic activities, excessive immune stimulation, and gene transfer. Over the period of 1990-2000 the consumption of *L. rhamnosus* GG increased substantially in Finland, however, infections due to probiotic strains were scarcely reported and no increase in bacteraemia caused by *Lactobacillus* species was seen[160]. Nevertheless, some probiotics have the ability to translocate and a small number of cases of infections have been traced back to consumption of *S. boulardii* and *L. rhamnosus* GG[161]. However, the vast majority of these patients was hospitalized and had an indwelling catheter. In addition, case reports have identified *S. boulardii* to cause fungemia in two immunocompromised patients[162], and exacerbation of diarrhea in two patients with ulcerative colitis[163].

Another safety issue is whether probiotics have the potential to permanently alter the composition of the intestinal microbiota. Tannock *et al.* (2005) suggested that it is unlikely that probiotics can significantly alter the intestinal microbiota since, even when administered in large quantities, the probiotic strain would only account for approximately 1% of the total bacterial count. However, numerous studies have indicated that probiotics can indeed alter the composition of intestinal microbiota in animals[164], and in humans[165], although in many cases, these alterations persist only shortly following cessation of probiotic administration. Accordingly long term probiotic administration may be required in certain settings.

Thus, despite reassuringly low infection rates for healthy humans, it will still be important to determine the safety profile for potential probiotic strains, including determination of strain resistance to a variety of common classes of antibiotics, and conformation of non-transmission of drug resistance genes. From an efficacy and safety perspective it would be preferable that probiotic bacteria exhibit a tolerance to antimicrobial agents used in clinical practice, that they do not transmit such resistance to other bacteria. Standardization of safety tests is an important task for the near future for researchers and companies in the field of probiotics.

Finally, the safety for the environment of new genetically modified probiotic strains should also be considered, and survival of genetically modified strains in the environment should be avoided. For instance, Steidler and colleagues (2000) addressed this issue in modified IL-10 producing *Lactococci* by replacing the *Lactococcus* gene for thymidilate synthase by the IL-10 transgene. This resulted in a strain that is dependent on the presence of thyminine or thymidine and thus unlikely to survive in the environment.

160 Isolauri *et al.*,2002
161 Hennequin *et al.*,2000; Land *et al.*,2005
162 Riquelme *et al.*,2003
163 Candelli *et al.*,2003
164 Gaudier *et al.*,2005
165 Cui *et al.*,2004

MULTISTRAIN, MONOSTRAIN, OR BACTERIAL COMPONENTS

As outlined in previous sections, probiotic strains exert their beneficial effects through a variety of mechanisms that are unique for each strain and obviously the choice of strain or combination of strains is crucial for therapeutic success. In studies on efficacy and mechanisms of action it is essential to keep in mind that *in vitro* effects do not necessarily correlate with probiotic actions *in vivo*. For instance, only a small minority of the lactic acid bacteria has the abilities needed for positive probiotic effects, i.e. survival through the harsh environment of the upper gastrointestinal tact, fermentation of fibers for nutrition, and the ability to eliminate unwanted pathogens[166]. Therefore mechanistic studies of probiotics must be performed by combining cell models and in *in vivo*-models, and its potential efficacy and safety verified in randomized controlled trials in patients.

The qualitative differences between strains imply that it may be possible to select monostrain or multistrain probiotics with the desired spectrum of abilities for use in different disease conditions. Monostrain probiotics are defined as a product that contains one single strain of a certain species, and consequently multistrain probiotics contain multiple strains from the same species or at least for the same genera. The term multispecies probiotics defines a group of products composed of multiple strains preferably from multiple genera.

It can be hypothesized that a combination of probiotic strains may complement or improve the health benefits given by individual strains. And indeed, an increasing number of scientific reports have appeared on the effects of probiotic combinations on the health of the host. The composition of the intestinal microbiota is partly dependent on individually determined host factors; therefore a multistrain probiotic may have more potential to colonize the intestinal lumen than a monostrain probiotic. Indeed, it has been shown that probiotics containing only one strain have a low probability of effectively colonizing the luminal niches of the gastrointestinal tract[167]. In addition, Collado and colleagues (2007) were able to show that all combinations among the probiotic strains tested (*Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* 99, and *Propionibacterium freudenreichii* ssp. *shermanii* JS), enhanced the adhesion properties of each separate probiotic strain, suggesting that that probiotic combinations could synergistically increase the beneficial health effects as compared with individual strains.

Furthermore, probiotics are used in the treatment of multi-factorial diseases demanding a variety of the probiotic properties, whereas such properties depend on the particular bacterial strain used. The frequently studied probiotic combination VSL#3 (consisting of a mixture of 8 lactic acid bacterial species), has been reported to be more effective in the treatment of pouchitis and ulcerative colitis as compared to conventional treatment strategies e.g. antibiotics, or monostrain probiotics[168]. The mechanisms by which multispecies mixtures synergistically enhance the probiotic effects remain obscure. Although suggested factors that play a role in enhancing the effect of multispecies probiotics are: 1) improving chances of successful colonization of at least one or several stains by e.g. reduction of antagonistic activity of the

166 Bengmark, 2005
167 Famularo *et al.*,1999
168 Ulisse *et al.*,2001; Gionchetti *et al.*,2002

endogenous microflora against other sensitive probiotic strain, induction of an optimal pH environment, creation of an anaerobic niche or enhancement of adhesion abilities; 2) enhancement of the combined effect due to strain specific properties e.g. synergistic effects of different strains regarding specific properties, enhancement of the total effect due to combination of several strain specific effects and symbiosis between probiotic strains due to exchange of nutrients[169].

In other words, there are a multitude of arguments in favor of a "group attack" by multistrain probiotics in the treatment of gastrointestinal pathophysiology. On the other hand, several recent studies have shown that soluble factors produced by probiotic strains may be an effective choice under certain circumstances. For example, conditioned media of VSL#3 inhibits NF-κB-activity and induces Hsp expression in epithelial cells[170], and recently Yan *et al.* (2007) showed that p40 and p75 proteins purified from *L. rhamnosus* GG conditioned media promotes colonic epithelial homeostasis through TNF-α-dependent pathways. These findings suggest that probiotic bacterial components may be isolated and used as a "magic bullet" for preventing and treating gastrointestinal disorders.

169 Timmerman *et al.*,2004
170 Petrof *et al.*,2004; Tao *et al.*,2006

THERE ARE A MULTITUDE OF ARGUMENTS IN FAVOR OF A "GROUP ATTACK" BY MULTISTRAIN PROBIOTICS IN THE TREATMENT OF GASTROINTESTINAL PATHOPHYSIOLOGY

MOST
ANIMAL STUDIES
ARE DESIGNED AS
PRETREATMENT
STUDIES,WHERE THE
PATHOPHYSIOLOGY
IS PREVENTED
RATHER THAN
CURED

CONCLUDING REMARKS

With the complex pathophysiology of gastrointestinal diseases it is difficult to obtain cure or total symptom relief. Treatment at various levels of pathophysiology and of numerous different symptoms is often necessary. Based on the studies reviewed here it is obvious that probiotics have potential positive effects on several of the pathophysiological events in stress-induced gut dysfunction (summarized in figure 1), and there is clearly a rationale for treating patients with stress-related intestinal disorders such as irritable bowel syndrome and inflammatory bowel disease. We are however far from being able to choose the precise combination of strains or bacterial components for each clinical setting. Therefore, even more emphasis is needed on studies of stress-related pathophysiology in humans and novel mechanisms of probiotic functionality.

A common observation is that the outstanding effects of probiotics in animal models of disease are difficult to repeat in the clinical setting. One important explanation may be that most animal studies are designed as pretreatment studies, where the pathophysiology is prevented rather than cured. Another common problem is that the interpretation of clinical studies is confounded by inhomogeneous patient groups, with various diseases and different degrees of physiological and immunological failure. Moreover, systematic reviews and meta-analyses sometimes lump together different probiotic strains and combinations of strains with prebiotics and synbiotics. In other words, to further elucidate the possible therapeutic potential of probiotics there is a great need to take, from experimental studies, carefully selected probiotic compounds to multicentre well-controlled disease-specific randomized studies in patients with stress-related intestinal disorders. Such studies are demanding regarding collaborative efforts and costs, but are essential for taking the therapeutic potential in probiotics from bench to bedside, and eventually helping patients suffering from stress-related gastro-intestinal diseases.

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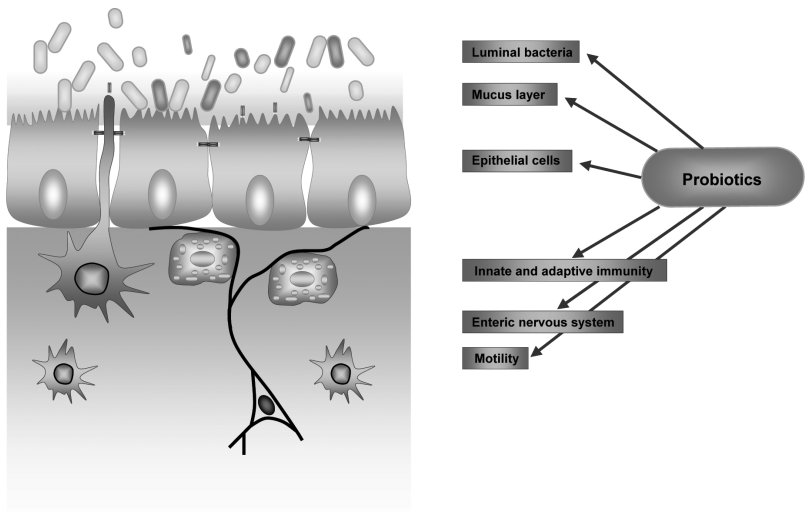
FIGURE 1

Overview of probiotic interactions with the host

Inside the lumen, probiotics interact with intestinal pathogens. Modulation of mucin secretion is one asset of the broad range of actions of probiotics to protect the mucosal barrier. At the epithelial level, cross talk between probiotics and enterocytes trigger a wide array of host genes involved in barrier function and defensin production. Probiotics also bind to Toll-like receptors on enterocytes and immune cells and exert immunomodulatory properties. There is a small but growing body of evidence that probiotics exert effects on the enteric nervous system. Probiotic modulation of the endogenous flora may influence transit through the gastrointestinal tract.



3

Enhanced translocation of bacteria across metabolically-stressed epithelia is reduced by butyrate.

Butyrate and epithelial permeability

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ABSTRACT

BACKGROUND

The gut microflora in some patients with Crohn's disease can be reduced in numbers of butyrate-producing bacteria and this could result in metabolic stress in the colonocytes. Thus, we hypothesized that the short-chain fatty acid, butyrate, is important in the maintenance and regulation of the barrier function of the colonic epithelium.

METHODS

Confluent monolayers of the human colon-derived T84 or HT-29 epithelial cell lines were exposed to dinitrophenol[1] + *E. coli*[2] ± butyrate[3]. Transepithelial resistance (TER), and bacterial internalization and translocation were assessed over a 24h period. Epithelial ultrastructure was assessed by transmission electron microscopy.

RESULTS

Epithelia under metabolic stress display decreased TER and increased numbers of pseudopodia that is consistent with increased internalization and translocation of the *E. coli*. Butyrate (but not acetate) significantly reduced bacterial translocation across DNP-treated epithelia but did not ameliorate the drop in TER in the DNP + *E. coli* exposed monolayers. Inhibition of bacterial transcytosis

1 DNP (0.1 mM), uncouples oxidative phosphorylation
2 strain HB101, 10^6 CFU
3 (3-50 mM)

across metabolically-stressed epithelia was associated with reduced IκB phosphorylation and hence NF-κB activation.

CONCLUSIONS

Reduced butyrate-producing bacteria could result in increased epithelial permeability particularly in the context of concomitant exposure to another stimulus that reduces mitochondrial function. We speculate that prebiotics, the substrate for butyrate synthesis, is a valuable prophylaxis in the regulation of epithelial permeability and could be of benefit in preventing relapses in IBD.

INTRODUCTION

Increases in intestinal epithelial permeability are associated with a variety of diseases including celiac disease, diabetes and inflammatory bowel disease (IBD)[4]. Moreover, when significant numbers of lumen-derived bacteria breach the epithelial barrier, via lesions or by permeating between (paracellular) or through (transcellular) the enterocytes, and enter the systemic circulation, the result can be sepsis, multi-organ failure and death[5]. Thus, the barrier function of the polarized epithelial cells lining the gut is an important first line of defense and appropriate regulation of the permeability characteristics of the epithelial layer is critical in homeostasis and host well-being.

Control of the paracellular and transcellular permeation pathways across the epithelium is an energy-dependent process, which led us to hypothesize that metabolic stress in the enterocyte would result in reduced barrier function. This postulate is based on a number of observations[6], including: the presence of swollen mitochondria in the epithelia and decreased ATP levels in the colon of some patients with Crohn's disease[7]; tissues excised from patients with Crohn's disease are more susceptible to the uncoupling of oxidative phosphorylation from the electron transport chain[8]; and, the increase in epithelial permeability caused by exposure to toxins from *Clostridium difficile* are preceded by derangements in mitochondrial structure and function[9]. Sources of metabolic stress in the intestine are varied and include psychological stress, medications, infections, ischemia and mucosal inflammation[10]. We have shown that monolayers of the crypt-like T84 epithelial cell line when exposed to dinitrophenol (DNP: uncouples oxidative phosphorylation) and non-pathogenic, non-invasive *E. coli* (strain HB101), displayed a significant drop in transepithelial resistance (TER), along with increased internalization and translocation of the bacteria, and a two-fold increase in IL-8 production[11]. Thus, the metabolically stressed enterocytes responded to the 'commensal' bacteria in a manner that is comparable to changes in epithelial function that are often seen in IBD[12].

Recently, dysbiosis of the colonic microflora has been described and implicated in the pathogenesis of IBD. Reductions in firmicutes, specifically, *Clostridium* XIVa and IV species have been shown in a small number of patients with Crohn's disease[13]; these bacteria are a major source of the short-chain fatty acid (SCFA), butyrate[14], which is the primary energy source for the colonocytes[15]. Thus, loss of butyrate-producing bacteria from the colonic microflora would be expected to result in a degree of metabolic stress and therefore increased epithelial permeability. The present study was designed to determine the impact of butyrate on epithelial

4 Laukoetter *et al.*, 2008
5 Turner, 2006
6 Lewis & McKay, 2009
7 Nazli *et al.*, 2004; Soderholm JD *et al.*, 2002; Schurmann *et al.*, 1999
8 Soderholm JD *et al.*, 2002
9 He *et al.*, 2000
10 Basivireddy *et al.*, 2002; Ramachandran *et al.*, 2001; Soderholm *et al.*, 2002; Ma *et al.*, 2006
11 Lewis et al., 2008; Nazli et al., 2004; Nazli et al., 2006
12 Xavier & Podolsky, 2007; Keita et al., 2008
13 Manichanh et al., 2006; Sokol et al., 2008; Frank et al., 2007
14 Barcenilla *et al.*, 2000
15 Roediger, 1980

THE PRESENT STUDY WAS DESIGNED TO DETERMINE THE IMPACT OF BUTYRATE ON EPITHELIAL BARRIER FUNCTION

WHEN BACTERIA BREACH THE EPITHELIAL BARRIER, THE RESULT CAN BE SEPSIS, MULTI-ORGAN FAILURE AND DEATH

barrier function. Using an *in vitro* model of metabolic stress + non-pathogenic *E. coli*-induced increases in epithelial permeability, we show that butyrate significantly reduces bacterial internalization and translocation across T84 cell monolayers, while having no effect on the drop in TER. The amelioration of enhanced bacterial translocation across epithelial monolayers was associated with inhibition of NF-κB activation in the enterocyte. Increasing the amount of glucose available to the cells also prevented the increase in bacterial translocation across metabolically-stress T84 cell monolayers.

MATERIALS AND METHODS

CO-CULTURE CELL MODEL

The human colon-derived T84 crypt-like cell line was used as a model epithelium throughout this study. T84 cells (passages 42-84) were seeded at a density of 10^6 cells/ml onto semi-permeable filters (3μm pore size, 1.1 cm^2 surface area; Costar, Cambridge, MA) or 12 well plastic culture plates and cultured at 37°C in a 1:1 mixture of Dulbecco's modified Eagle medium and Hams F-12 medium, supplemented with 2% pen-strep, 1.5% HEPES, and 10% fetal bovine serum. Filter grown monolayers were considered confluent when TER was >750Ω/cm^2 (monolayers used in these studies had starting TER values of 750-3030 Ω/cm^2) [16] In some experiments monolayers of the HT-29 human colon-derived epithelial cell line (TER range 170-250Ω/cm^2) were used. Plastic-grown T84 cell preparations were typically used at ~70% confluence as determined by phase-contrast microscopy. *Escherichia coli* strains HB101 and F18 as well as *Salmonella typhimurium* (a gift from Dr. M. Surette, Univ. Calgary) were grown and cultured overnight in an orbital shaker in Luria-Bertani (LB) broth at 37°C. *S. typhimurium* was diluted to a 1:33 ratio, and then allowed to grow for another 3h in the shaker. Bacteria (10^6 colony forming units (CFU)) in the stationary growth phase were added to the apical side of confluent T84 monolayers. Sodium-butyrate and 2,4-dintrophenol (DNP; 0.1mM) were purchased from Sigma Chemical Co. (St. Louis, MO). In some experiments acetate (Sigma Chemical Co.) was used instead of butyrate. In additional experiments, glucose, mannitol or O-methyl-D-glucopyranose (all at 25 mM; Sigma Chemical Co.) were added to the culture well. Putative direct toxic effects of butyrate (3, 20 or 50 mM) on the *E. coli* was assessed in 24h growth curves, where bacterial growth was determined by optical density readings and dilution plating analysis on agar plate and counting CFU.

EPITHELIAL MITOCHONDRIAL ACTIVITY

Mitochondrial activity was evaluated by measuring the mitochondrial-dependent reduction of colorless 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a colored blue formazan. T84 cells ($1x10^5$) were seeded in 96-well plates and after reaching confluence, were exposed for 2, 4 or 24h to DNP (0.1 mM), butyrate (3 mM), DNP+butyrate or staurosporine (5 μM). Subsequently, culture medium was aspirated from the wells, the cells washed with phenol red-free RPMI medium, supplemented with FBS (10%) and HEPES (36 μM) and MTT (100 μM) added for 4h at 37°C. The reaction was stopped by the addition of 50 μl of acidic isopropanol, absorbance measured at 595nm and results expressed in arbitrary units (*E. coli* was not included in these experiments because they metabolize MTT and mask the effects of DNP).

16 Lewis *et al.*, 2008

EPITHELIAL VIABILITY

As a marker of cell viability, the amount of lactate dehydrogenase (LDH) released into the culture medium was determined in a colorometric assay following the manufactures instructions (CytoTox96(R) Non-Radioactive Cytotoxicity Assay, Promega Corp., Madison, WI, USA). Staurosporine treated cells (5 μM, 24h) were used as a positive control.

EPITHELIAL BARRIER FUNCTION

i) Transepithelial Electrical Resistance (TER). T84 cells were seeded onto semi-permeable filters supports and TER an accepted index of paracellular permeability[17], was recorded before, and at 2, 4, 6, 12, 16, 24 and 48 h after treatment using a voltmeter and matched electrodes (Millicell-ERS; Millipore, Bedford, MA). Data were normalized to pretreatment TER and are expressed as the percentage of pretreatment values.

ii) Bacterial translocation. T84 cells were grown to confluence on 3.0 μm pore-size filters, transferred to antibiotic-free medium, and inoculated apically with *E. coli* HB101 or F18 (10^6 CFU) ± DNP (0.1 mM) ± butyrate (3 mM). Ten μl aliquots of culture medium were collected from the basolateral compartment 4 and 24 h later, and the presence of bacteria was determined by serial dilution and enumeration of bacterial colonies following 24h of culture at 37°C on LB agar plates. Alternatively, bacterial translocation was assessed using a previously validated semi-quantitative logarithmic 5-point scale[18]. 0 = no colonies; 1 = <10 colonies; 2 = 10-100 colonies; 3 = >100 countable colonies; 4 = >100 discrete colonies but uncountable; 5 = bacterial lawn.

BACTERIAL INTERNALIZATION

Semi-confluent monolayers of T84 cells were grown in 12-well plates and inoculated with *E. coli* HB101 or F18 or *S. typhimurium* ± DNP ± butyrate and incubated for 4h at 37°C. A sample of culture medium was collected, and the number of bacteria determined using the method of Miles and Misra[19]. Epithelia were washed extensively and treated with gentamicin (200 μg/ml) for 1.5h and then rinsed with sterile phosphate-buffered saline, lysed with cold Triton X-100 (20 min at 4°C), and plated onto LB agar. The number of internalized, viable bacteria was determined and is presented as a percentage of the total number of extracellular bacteria after a 4 h growth period. In some experiments, T84 cells were left in gentamicin for 4, 8 or 24h to assess the viability of internalized bacteria after an initial 4h period of bacterial contact.

17 McKay *et al.*, 2007
18 Lewis *et al.*, 2008
19 Lewis *et al.*, 2008

TRANSMISSION ELECTRON MICROSCOPY (TEM)

T84 cells were seeded into 3 cm Petri dishes and grown to ~70% confluence. Following exposure to *E. coli* + DNP ± butyrate, human recombinant tumor necrosis factor α (10 ng/ml; R&D Systems Inc.) ± LPS (*E. coli*-derived 1 μg/ml; Sigma Chemical Co.) for 6h or to antibiotic-free media alone, the cells were fixed in gluteraldehyde, processed and examined by TEM[20]. Images (6-15/monolayer) were captured and morphometric analysis (i.e. number and length of pseudopodia/20 μm of plasmalemma) was performed in a blinded fashion using coded prints.

IMMUNOBLOTTING

Whole cell protein extracts of T84 cells were assessed for total and phosphorylated-IκB expression. Briefly, cells were lysed in 200 μl of ice-cold lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM NaVO3, complete ETDA-free protease inhibitor complex (Roche Diagnostics, Indianapolis, IN), the extracts were sonicated 3 times for 10 sec, centrifuged at 13,000 rpm for 5 min at 4°C, and then stored at -80°C. After protein concentrations were determined using the Bio-Rad/Bradford assay, 40 μg of protein was mixed with loading buffer (2% (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, 100 mM dithiothreitol, (DTT) 1% (w/v) normophenol blue, 5% (v/v) glycerol) and run in a 10% polyacrylamide gel (100V for 1 h), and the separated proteins electroblotted onto polyvinylidene diflouride (PVDF) membranes (VWR). Membranes were blocked with 5% BSA for 2h at room temperature, followed by a 4°C overnight incubation with rabbit IgG against phospho-IκB, total IκB, IKK, p-IKK (1:1000; Cell Signaling Technology, Beverly, MA), Cullin-1, or NEDD8 (1:1000, Zymed Laboratories, San Francisco CA). Membranes were washed extensively, incubated with the appropriate secondary antibody (1 h at 1:5000 for p-IκB and 1:7000 for IκB, IKK, p-IKK, or overnight at 1:7000 for Cul-1 and NEDD8; Santa Cruz Biotechnology, Santa Cruz, CA). After washing, immunoreactive proteins were visualized by enhanced chemiluminescence (ECL: Amersham Pharmacia, Piscataway, NJ) and exposed to Kodak XB film (Eastman Kodak Co., Rochester, NY). Blots were scanned and the density of the bands calculated using Image J software (National institutes of Health, Bethesda, MD).

ELISA FOR IκB

The level of p-IκB ELISA in 40 μg of whole cell protein exact was measured by a commercial ELISA following the manufactures instructions (Medicorp Inc., Montreal, QB, Canada). The absorbance (optical density in arbitrary units) of each sample was determined spectrophotometrically using a microplate reader (Perkin-Elmer).

20 Nazli *et al.*, 2004

DATA PRESENTATION AND ANALYSIS

Data for TER, bacterial internalization, densitometry and spectrophometric analysis are presented as the mean ± standard error of the mean (SEM), where n values represent the number of individual epithelial monolayers from a certain number of indicated experiments. Statistical analysis was done using a one-way ANOVA followed by a post-hoc Tukey's test for pair-wise comparisons. Data from bacterial translocation studies were assessed using Pearson-chi^2 analysis. The p-value for each level of translocation was then assessed using a chi-goodness of fit test. A statistically significant difference in al tests was accepted at $p<0.05$.

RESULTS

DNP DISRUPTS MITOCHONDRIAL STRUCTURE AND EVOKES INCREASED MEMBRANE RUFFLING

Control, non-treated T84 cells contained numerous electron-dense mitochondria with well-defined cristae (fig 1A). In contrast, and as would be predicted based on the its ability to block oxidative phosphorylation, treatment of plastic-grown T84 cells with DNP for 6h resulted in swollen mitochondria with irregular and reduced cristae (fig 1B); comparable changes were observed in T84 cells treated with DNP+*E. coli* (fig 1C). This perturbation of mitochondrial activity was confirmed by reduced cleavage of the MTT substrate in T84 cells that had been treated with DNP 24h previously (fig 2). In addition, metabolically-stressed cells displayed an increase in the number of pseudopodia of 0.5-1.0 μm and >1 μm in length. This was further exaggerated (i.e. ~20-fold increase over control levels), and statistically significant, in enterocytes treated with DNP+*E. coli* (fig 3). In contrast, treatment with *E. coli* (HB101) alone, TNF-α (10 ng/ml) or TNF-α+LPS (1 μg/ml) did not elicit an increase in the number of pseudopodia of either size in T84 cells (fig 3). Another ultra-structural feature of DNP+*E. coli* treated cells, but not the other treatment groups, was obvious 'vesicle-type' structures which may be actual vesicles that have budded off the cells (perhaps analogous to exosomes[21]) or alternatively they could be obliquely sectioned pseudopodia (fig 1C). Butyrate (3mM) addition to the DNP+*E. coli* co-treatment regimen led to greater mitochondrial activity (fig 2) and significantly reduced the number of pseudopodia (fig 3); the extracellular 'vesicles' were also less prominent (fig 1D). Butyrate treatment resulted in the appearance of semi-circular, rounded mitochondria, often with evidence of a cytoplasmic inclusion, which has been associated previously with the biogenesis of mitochondria (fig 1D).

BUTYRATE BLOCKS THE INCREASED BACTERIAL TRANSCYTOSIS CAUSED BY METABOLIC STRESS

Addition of butyrate (0.1-100 mM) to confluent monolayers of T84 cells for 48 h did not lead to an increase in TER; indeed, ≥20 mM butyrate caused a significant decrease in TER of ~50% (fig 4A). Butyrate (3 mM) added to filter-grown T84 cells 3 days after seeding did not enhance the formation of a confluent monolayer as gauged by TER (data not shown). As previously shown[22], *E. coli* HB101 (10^6 CFU) when added to T84 cell monolayers alone did not cause a consistent significant decrease in TER. This was also true for the human fecal commensal *E. coli,* strain F18 (fig 4B). However, simultaneous addition of DNP (0.1 mM) and either *E. coli* HB101 or F18 caused marked decreased in TER that were unaffected by the addition of butyrate to the co-culture well (fig 4B, data for *E. coli* HB101 not shown). The drop in TER could reflect epithelial cell death via apoptotic or necrotic processes. We previously found no evidence of increased caspase 3 cleavage in DNP+*E. coli* treated T84 cells[23]. A lack of cell death was corroborated in the present

21 van Niel *et al.*, 2001
22 Philpott *et al.*, 1996
23 Lewis *et al.*, 2008

studies by LDH measurements that revealed no differences between controls (normalized to 100%) and DNP+*E. coli* treated (95±8% of control values) filter-grown T84 cell monolayers (n=3 monolayers) (similarly, increased laddering of DNA extracts of DNP+*E. coli* treated T84 cells was not observed (pers. obser.))

Exposure of T84 or HT-29 cell monolayers to DNP+*E. coli* HB101 resulted in a significant increase in bacterial translocation across the monolayers compared to *E. coli*-only treated epithelia: for example, in HT-29 cell monolayers exposed to DNP+*E. coli* 55% (i.e. 5 of 9) of the preparations showed a bacterial translocation level of 3 (i.e. between 11-100 CFU) and 0 of 9 monolayers shown no bacterial translocation, which was in contrast to *E. coli* only treated cells were 0 or 6 monolayers had a bacterial translocation score of 3. Moreover, and in contrast to TER, the enhanced translocation of *E. coli* across DNP co-treated T84 cell monolayers was significantly reduced by butyrate at 3 and 50 mM (fig 4C, D). Every T84 cell monolayer exposed to *E. coli* (HB101 or F18) + DNP for 4h showed some level of bacterial translocation and simultaneous addition of butyrate with the DNP+*E. coli* reduced the levels of bacterial translocation. For example, 65% of the butyrate+DNP+*E. coli* (HB101) treated monolayers displayed no bacterial translocation (fig 4C). Similarly, butyrate (50 mM) reduced *E. coli* (HB101) translocation across DNP-co-treated monolayers (e.g. 83% of monolayers treated with DNP+*E. coli* showed some degree of bacterial translocation 8h after treatment, whereas only 17% of DNP+*E. coli*+butyrate treated monolayers displayed bacterial translocation (n=6 monolayers from 2 experiments)).

Inhibition of bacterial translocation could be due to either a toxic effect of butyrate on the *E. coli* or another butyrate effect on the bacterium. We have data in support of neither possibility, since 24h growth curves for either *E. coli* HB101 or F18 were identical in the presence of 3, 20 or 50 mM butyrate (n=2-3) and *E. coli* exposed to butyrate for 3h and then washed showed a similar enhancement of transcytosis across DNP-co-treated T84 monolayers as those not treated with butyrate (data not shown). Also substantial bacterial translocation occurred by 4h post-DNP+*E. coli* treatment (i.e. 10^3-10^5 CFU/ml), when TER was not significantly reduced, remaining at 89±10% of pretreatment values (n=6 T84 cell monolayers).

By 24h post-treatment the effect of butyrate had waned and differences between DNP+*E. coli* and butyrate+DNP+*E. coli* treated T84 cell monolayers were no longer evident (data not shown). However, when butyrate was administered every 4h over a 24h period and bacterial translocation assessed at the end of the 24h treatment period, almost 40% of T84 cell monolayers displayed no bacterial translocation whereas every epithelial monolayer treated with DNP+*E. coli* showed translocation levels at ≥grade 2 (fig 4E). Repeated butyrate treatments did not affect the drop in TER observed 24h after exposure to DNP+*E. coli* (data not shown).

Using the 2-carbon SCFA, acetate, as a comparator molecule, we observed no bility of this molecule to ablate the enhancement of *E. coli* translocation across metabolically stressed T84 cell monolayers (n=6 epithelial preparations from 3 experiments; data not shown).

BUTYRATE BLOCKS THE INCREASED BACTERIAL INTERNALIZATION CAUSED BY METABOLIC STRESS

Bacteria can cross the epithelium by passing between the tight junctions or via a transcellular route. Therefore, bacterial internalization into the enterocytes was assessed using the gentamicin protection assay. As shown in figure 5, internalization of *E. coli* by T84 cells was significantly increased 4h post co-treatment with DNP and this was reduced by butyrate treatment. Given the relatively low levels of bacterial internalization, experiments were conducted with invasive pathogen *Salmonella typhimurium*. The same pattern was observed; increased intracellular *S. typhimurium* in DNP-treated T84 cells, which was reduced in the presence of butyrate (fig 5C).

Increases in the number of intracellular bacteria could be due to either an increased rate of internalization or a decreased ability of the metabolically-stressed epithelia to kill the internalized bacteria. To test these possibilities, T84 cells were treated for 4h with DNP+*E. coli*±butyrate, then treated with gentamicin for 4, 8 or 24h, at which point the cells were lysed and viable bacteria enumerated. Using this approach, by 24h the number of intracellular bacteria was similar in all conditions, suggesting that there was increased internalization and not simply a reduced ability of the enterocytes to kill bacteria (fig 5D).

BUTYRATE PREVENTS DNP+*E. COLI*-INDUCED NF-κB ACTIVATION VIA INHIBITION OF IκB

Since pharmacological inhibitors of NF-κB activity reduce bacterial transcytosis across DNP-treated T84 cell monolayers[24], we postulated that the effects of butyrate could be mediated by the inhibition of NF-κB activation. Immunoblotting revealed that butyrate reduced the increase in phosphorylated-IκB levels observed in T84 cells exposed to DNP+*E. coli* (fig 6). These findings were corroborated by use of an ELISA for phosphorylated-IκB (fig 6C).

Phosphorylation of IκB occurs via the activity of the enzyme, IKK, whose activity is also dependent on phosphorylation of serine in the case of IKKκ and serine in the case of IKKκ. Immunoblotting detected no change in the level of phosphorylated-IKK when extracts from DNP+*E. coli* were compared with those from time-matched butyrate+DNP+*E. coli* treated T84 cells (n=3). Ubiquitination of phosphorylated-IκB occurs when the ubiquitin-like conjugating enzyme (Ubc-12) neddylates the Cullin-1 subunit of E3-SCF (the enzyme responsible for ubiquitinating p-IκB). Under the experimental conditions employed here, butyrate-induced alterations in NEDD8-Cullin-1 conjugation were not observed (n=4).

INCREASING EXTRACELLULAR GLUCOSE INHIBITS THE INCREASED BACTERIAL TRANSLOCATION ACROSS METABOLICALLY-STRESSED T84 CELL MONOLAYERS BUT NOT THE DROP IN TER

Increasing the amount of extracellular glucose in the medium from 12.5 to 25 mM had no effect on the drop in TER caused by exposure to DNP+*E. coli*, indeed the drop in TER was statistically significantly greater in glucose+DNP+*E.*

24 Lewis *et al.*, 2008

coli treated T84 cells at 24h post-treatment (fig 7A). However, T84 cell monolayers provided additional glucose showed less bacterial transcytosis as a consequence of exposure to DNP+*E. coli* (assessed 4h after treatment; fig 7B). This was not due to an osmotic effect since equi-molar mannitol could not substitute for glucose. Neither was it via activation or opening of glucose transporters because 3-O-methyl-D-glucopyranose, which is taken up with similar kinetics to glucose but cannot be metabolized, also did not reproduce the effect seen with glucose (fig 7B).

FIGURE 1

DNP disrupts mitochondrial structure and evokes increased membrane ruffling

T84 cells exposed to 2,4-dinitrophenol (DNP) contain swollen and mis-shapen mitochondria. Transmission electron microscopy images of control, non-treated (A), DNP (0.1 mM)-treated (B), DNP+*E. coli* (strain HB101, 10^6 CFU)-treated (C) and DNP+*E. coli*+butyrate (3 mM)-treated (D) T84 cells. Cells were grown on plastic culture-area and treated for 6h. Images are representative of 3 monolayers/per condition (10-20 sections/T84 cell monolayer) (black arrowheads depict normal mitochondria, white arrowheads highlight swollen mitochondria with irregular cristae, black arrows point to pseudopodia, white arrows point to 'vesicle-like' structures, N=nucleus and *=putative regenerating mitochondria)

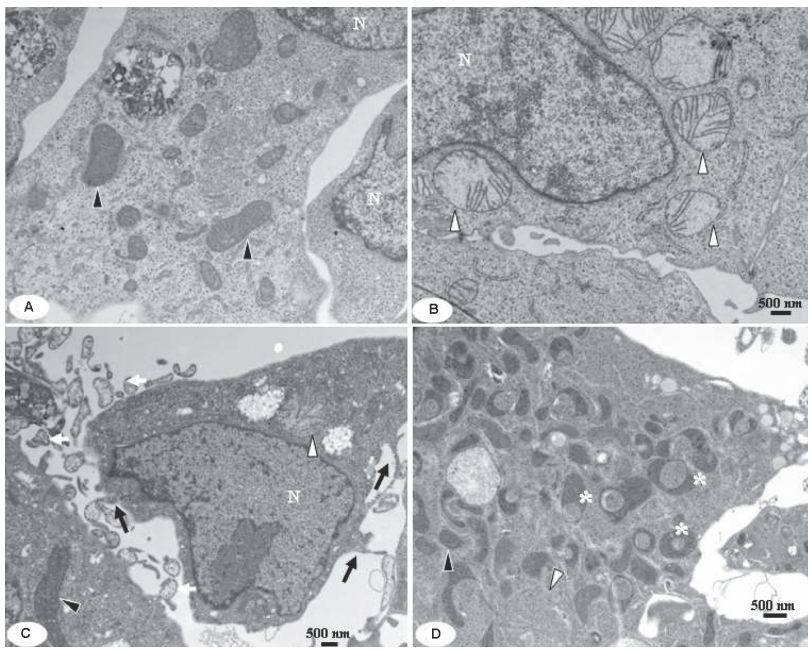


FIGURE 2

Butyrate prevents DNP-induced reduction in mitochondrial activity

Bar graph showing that exposure of T84 epithelial cells to DNP (0.1 mM) results in reduced mitochondrial activity as gauged by MTT cleavage and that this is not observed in butyrate (3 mM) co-treated cells (data are mean±SEM; n=8 epithelial preparations from 2 experiments; * and #, $p<0.05$ compared to time-matched control and DNP only, respectively; staurosporine was included as a positive control (5 μM)).

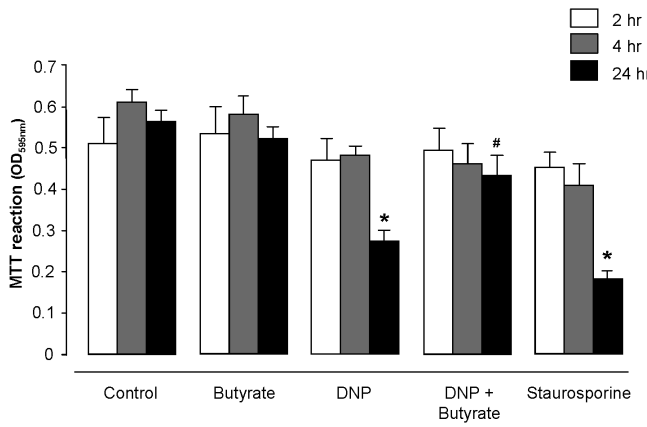


FIGURE 3

Butyrate reduces DNP+*E. coli*-induced pseudopodia formation

Bar graph showing that exposure of T84 epithelial cells to DNP (0.1 mM) + *E. coli* (HB101) results in pseudopodia formation that is reduced by butyrate (3 mM) co-treatment (data are mean±SEM; n=18-57 cells for each condition; *$p < 0.05$ compared to control; pseudopodia were counted and measured on transmission electron microscopy images (see fig 1) of plastic-grown T84 cells exposed to the various treatments for 6h; TNF-α, human recombinant TNF-α at 10 ng/mL; LPS, *E. coli*-derived lipopolysaccharide at 1 μg/ml).

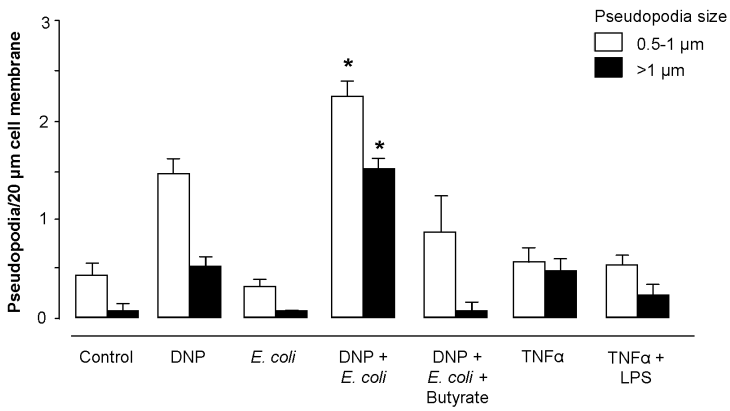


FIGURE 4

Increased epithelial transcytosis of *E. coli* across metabolically stressed T84 epithelial cell monolayers is reduced by butyrate co-treatment

(A) Bar graph showing that 24 or 48 h exposure to butyrate does not increase transepithelial resistance (TER), rather diminishing it at doses of 20 mM or greater (n=4-14 T84 cells monolayers from 7 experiments). (B) Similarly butyrate (3mM) does not prevent the decrease in TER caused by 24h exposure to DNP (0.1 mM) and *E. coli* (HB101) (n=14 T84 cells monolayers from 7 experiments). In contrast, butyrate (3 mM (Panel C) or 50 mM (Panel D)) reduced *E. coli* HB101 translocation across DNP-co-treated monolayers (n=16-21 monolayers from 7 experiments (panel C) or n=6 monolayers from 2 experiments (panel D); bacterial transcytosis was assessed 4h post-DNP+*E. coli*±butyrate co-treatment). Panel E shows that addition of butyrate (3 mM) every 4h into the co-culture sustains the inhibition of bacterial translocation until 24h post-treatment (end of exp) (n=6-9 monolayers from 3 experiments) (data are mean ± SEM; *, $p<0.05$ compared to control).

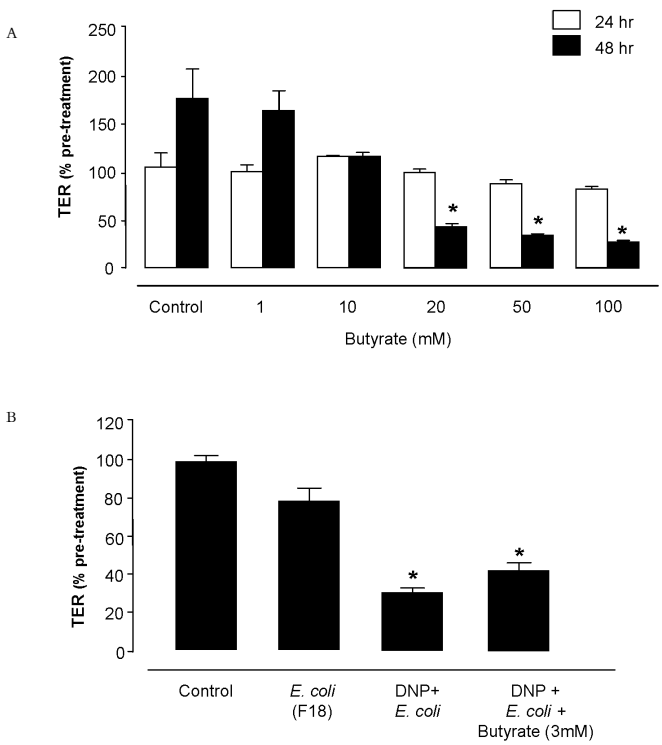


FIGURE 4

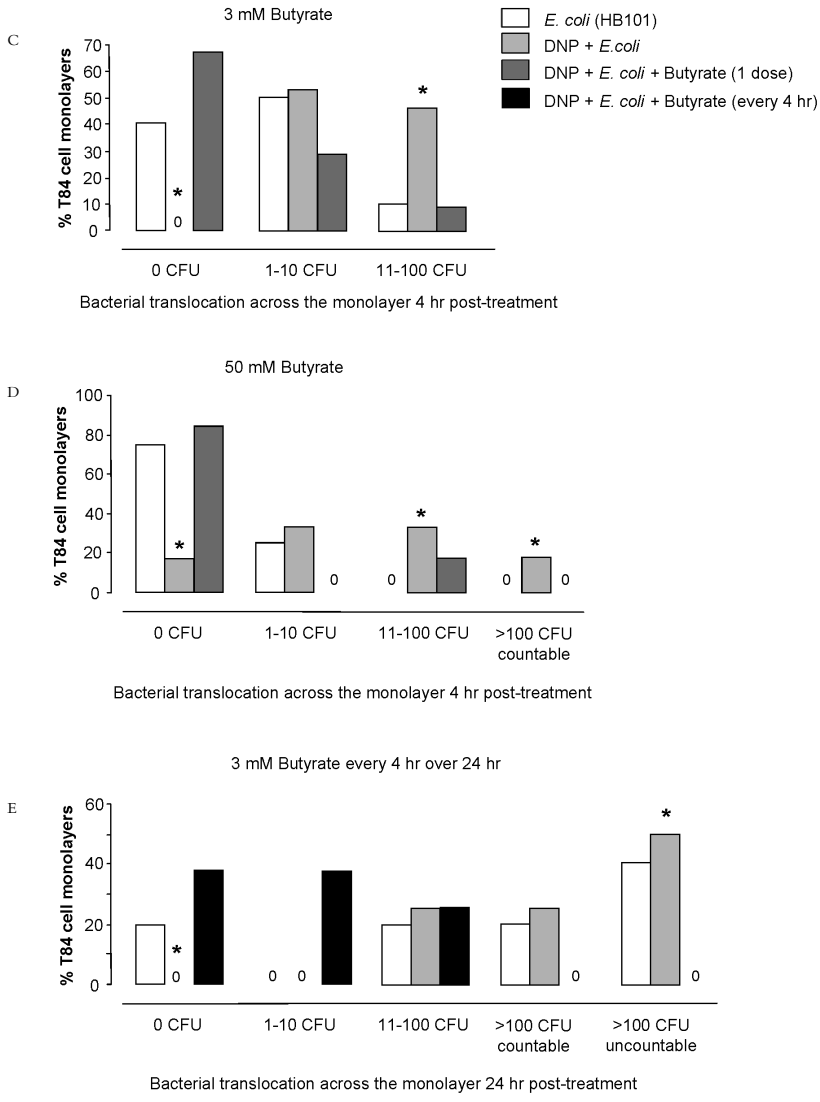

C
3 mM Butyrate
E. coli (HB101)
DNP + E.coli
DNP + E. coli + Butyrate (1 dose)
DNP + E. coli + Butyrate (every 4 hr)
% T84 cell monolayers
0 CFU
1-10 CFU
11-100 CFU
Bacterial translocation across the monolayer 4 hr post-treatment
D
50 mM Butyrate
% T84 cell monolayers
0 CFU
1-10 CFU
11-100 CFU
>100 CFU countable
Bacterial translocation across the monolayer 4 hr post-treatment
E
3 mM Butyrate every 4 hr over 24 hr
% T84 cell monolayers
0 CFU
1-10 CFU
11-100 CFU
>100 CFU countable
>100 CFU uncountable
Bacterial translocation across the monolayer 24 hr post-treatment

FIGURE 5

Butyrate co-treatment prevents the increase in bacterial internalization

Bar graph showing that butyrate (3 mM) co-treatment prevents the increase in *E. coli* HB101 (A), *E. coli* F18 (B) and *Salmonella* (C) internalization into DNP (0.1 mM)-treated epithelia and invasion by *S. typhimurium* (n=6-20 T84 cell preparations from 4-5 experiments; internalization assays conducted 4h after exposure to the experimental conditions). Panel D shows that DNP (0.1 mM) does not inhibit the ability of T84 epithelial cells to kill internalized bacterial, since levels of intracellular *E. coli* are similar at 24h after extracellular bacteria are killed by treatment with gentamicin to prevent any further internalization of viable *E. coli* (data are mean±SEM; *, $p<0.05$ compared to bacteria (10^6 CFU) only).

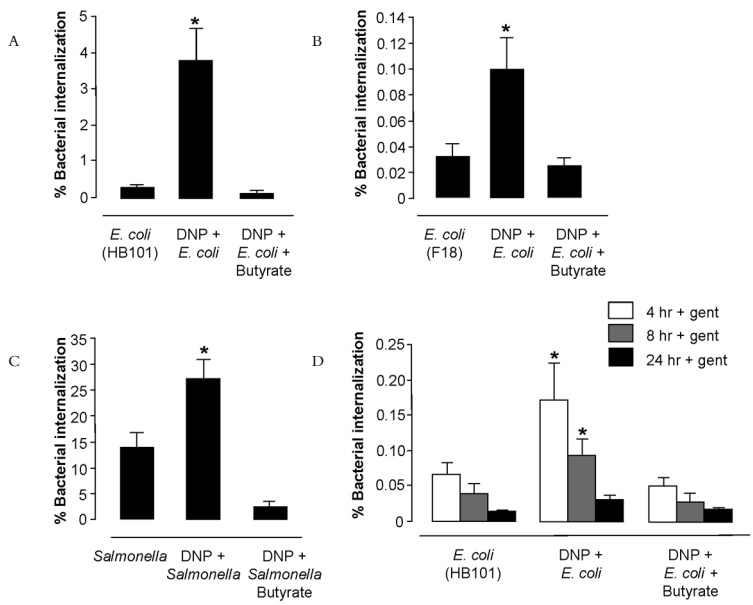


FIGURE 6

Butyrate inhibits T84 epithelial cell mobilization of NF-κB in response to treatment with DNP+*E. coli*

Panel A are representative immunoblots from whole cell lysates collected at 45 and 60 min after treatment (con, control; D, DNP at 0.1 mM; E, *E. coli* at 10^6 CFU; B, butyrate at 3 mM; X, blank lane; p-IκB, phosphorylated IκB). Panel B, densitometry of immunoblots reveals a statistically significant increase in the IκB:p-IκB in response to DNP+*E. coli* that is inhibited by co-treatment with butyrate (data are mean±SEM; n=3-9 T84 cell preparation from 4 experiments; *, p<0.05 compare to other groups). (C) ELISA for the phosphorylated form of IκB supports the analysis of immunoblotting shown in panel B (n=3-9 T84 cell preparations from 4 experiments; *, p<0.05 compared to control; TNF-α used as a positive control at 10 ng/mL for 24h (n=6 epithelial preparations)).

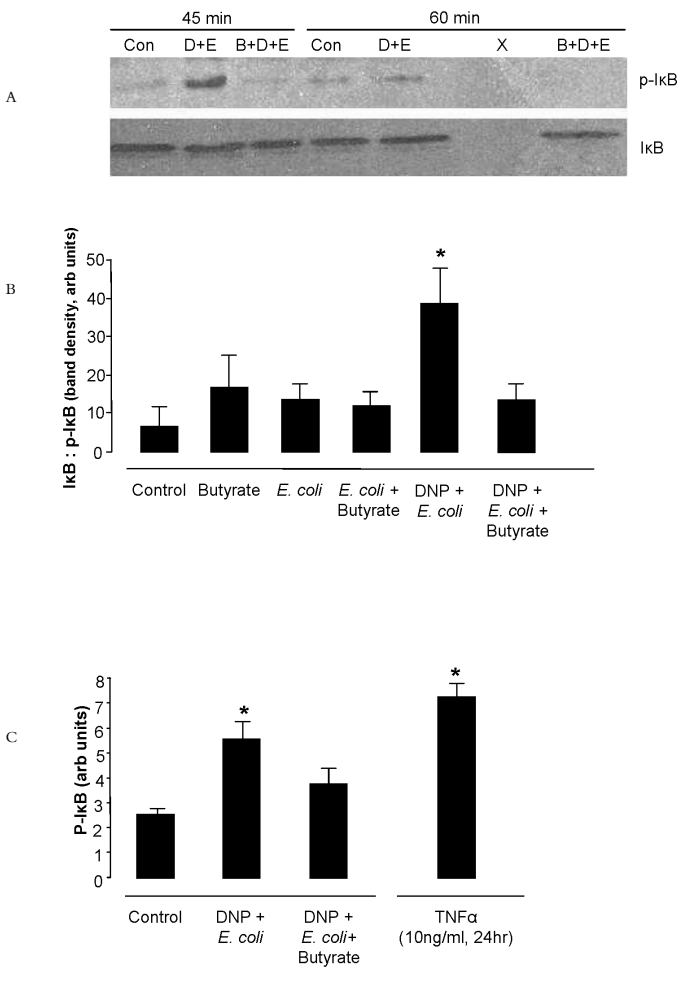
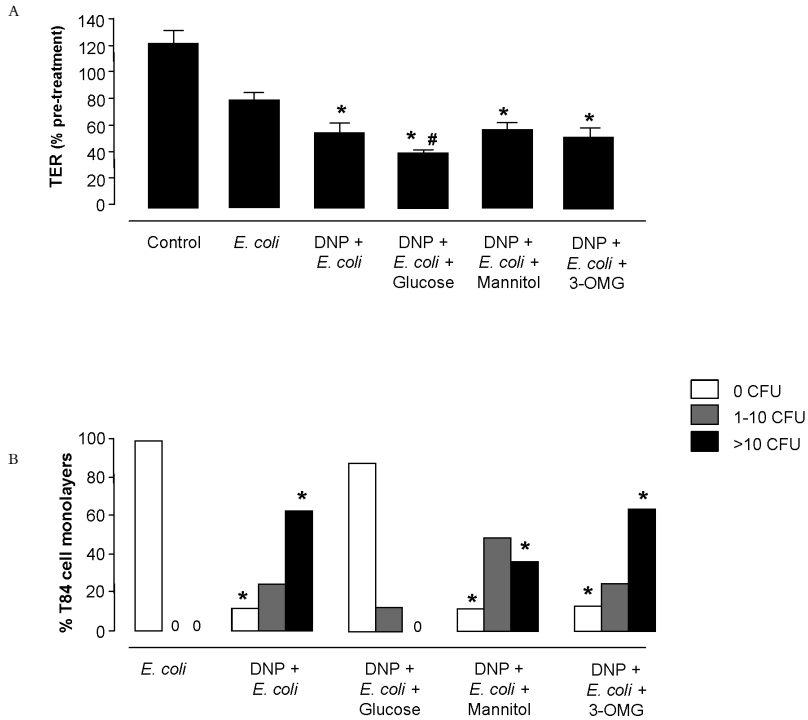


FIGURE 7

Glucose prevents the DNP+*E. coli*-induced bacterial translocation

Bar graph (A) showing that increasing the amount of extracellular glucose to 25 mM did not prevent the drop in transepithelial resistance caused by a 24h treatment of DNP (01. mM) + *E. coli* (HB101, 10^6 CFU). In contrast, increased glucose inhibited the enhanced bacterial translocation across confluent T84 cell monolayers (panel B), and this was not observed when equi-molar mannitol or the non-absorbable 3-O-methyl-D-glucoprranose (3-OMG) was substituted for glucose (data are mean±SEM; n=7-8 monolayers from 3 experiments for each condition; Panel A: ⋆, $p<0.05$ compared to control; #, $p<0.05$ compared to DNP+*E. coli*; Panel B: bacterial transcytosis was assessed 24h post-DNP+*E. coli* treatment and a 0 indicate no translocation, ⋆, $p<0.05$ compared to DNP+*E. coli*+glucose).



DISCUSSION

The epithelial cells of the gastrointestinal tract stand as guardian and sentinel at the boundary between the body and the myriad of antigens and microbes that occur in the gut lumen. Thus, the efficient control of epithelial permeability is of paramount importance to health[25]. Reduced epithelial mitochondrial activity can accompany decreased barrier function provoked by, for example, infection, or psychological stress[26]: an intuitive point since regulation of the tight junctions and endocytosis are energy-dependent processes. Postulating that metabolic stress in the enterocyte could be common to a variety of stimuli that result in decreased epithelial barrier function that could contribute to the genesis of IBD or provoke relapses in disease activity, we showed increased T84 cell monolayer permeability 24h after treatment with DNP but only when non-invasive, non-pathogenic commensal *E. coli* were also present[27]: an intriguing observation given that a normal gut microflora may be a prerequisite for the development of colonic inflammation[28].

Altered composition of the gut microflora is common in patients with IBD and recent studies suggest a loss of butyrate-producing bacteria[29]. The consequence of this is unclear but since ~70% of a colonocyte's energy is derived from butyrate[30], reductions in the number of butyrate-producing bacteria could result in focal metabolic stress. Therefore, in theory, butyrate could reduce the increases in epithelial permeability evoked by metabolic stress elicited by other stimuli. Indeed, metabolic stress and perturbed butyrate metabolism has been postulated as a cause of ulcerative colitis[31]. Recently, reduced expression of a butyrate transporter, namely the monocarboxylate transporter 1 (MCT1), was shown in biopsies from patients with ulcerative colitis or Crohn's disease, tissue from mice with dextran sodium sulfate-induced colitis and in the human colon-derived HT-29 epithelial cell line treated with IFN-$\gamma\pm$TNF-α[32]. Thus, colonocytes can experience metabolic stress as a consequence of reduced production of butyrate by the microflora, a reduced capacity to absorb the SCFA, or via perturbed β-oxidation of butyrate and improper metabolism.

Initial studies supported earlier findings of reduced mitochondrial activity in DNP-treated T84 cells and that this treatment did not result in increased cell death[33]. In addition, cells co-treated with DNP+*E. coli* displayed enhanced membrane ruffling as shown by increased pseudopodia, which is consistent with the increased internalization of *E. coli*. Addition of low dose (concentration in the gut ranges 70-100 mM[34]) butyrate to the cell cultures restored mitochondrial activity and resulted in numbers of pseudopodia that were not different from control values. Furthermore, while the drop in TER observed after exposure to DNP+*E. coli* was

25 Deitch *et al.*, 2006; De-Souza & Greene, 2005
26 Lewis & McKay, 2009
27 Nazli *et al.*, 2004
28 Sartor, 2008
29 Manichanh *et al.*, 2006; Sokol *et al.*, 2009
30 Cook & Sellin, 1998; Miller, 2004
31 Roediger, 1980; Santhanam *et al.*, 2007
32 Thibault *et al.*, 2007
33 Nazli *et al.*, 2004; Nazli *et al.*, 2006
34 Miller, 2004

unaffected by single or multiple treatments of butyrate, the increased internalization of *E. coli* (and *S. typhimurium* invasion) and their transcytosis across T84 (or HT-29) epithelial cell monolayers was significantly reduced. In fact a significant amount of bacterial translocation occurred in the absence of reductions in TER, indicating that the paracellular and transcellular permeation pathways are differentially regulated, with the latter being a route that bacteria can use to breech the epithelial barrier, and which is regulated by butyrate.

Enteroinvasive *E. coli* have been associated with Crohn's disease[35], and the data herein clearly show that metabolic stress can facilitate the uptake and transcytosis of non-invasive *E. coli* across an epithelial cell layer. The commensal gut flora provide a range of benefits for their host[36]. Production of butyrate by the commensal microflora could be an extrinsic defense mechanism to limit the uptake of lumen-derived antigen and microbes, which would be particularly important following exposure to stimuli that perturb mitochondrial function.

A substantial body of evidence shows that butyrate is anti-inflammatory[37], blocking, amongst other things, the mobilization of NF-κB and subsequent pro-inflammatory events[38]. Pharmacological analysis implicated NF-κB signaling in the DNP+*E. coli*-induced increases in bacterial transcytosis (but not the drop in TER)[39]. T84 cells exposed to DNP+*E. coli* show increased phosphorylation of IκBα that would result in its' degradation and subsequent activation of NF-κB: this response to DNP+*E. coli* was significantly reduced in butyrate-treated epithelia. Additional experiments revealed neither reduced IKK activation (the enzyme that phosphorylates IκBα) nor reduced neddylation of Cullin-1, that is critical in E3 ligase activity. The latter appears to contradict data from Kumar *et al.* (2009) who showed deneddylated Cullin-1 in butyrate-treated T84 cells. However, in that study, the pH of the culture medium was 4.5-5.0 which could result in protonation and diffusion of butyrate through the plasmalemma. This raises the possibility that inhibition of NF-κB in epithelia by butyrate may be dependent on signaling events specific to how butyrate enters the cell and its oxidation status[40]. While our data are consistent with butyrate inhibition of NF-κB signaling and a concomitant reduction in bacterial translocation across metabolically stressed epithelia, we must be mindful of the possibility that butyrate is not a specific inhibitor of NF-κB and would be expected to simultaneously affect other signaling systems in the enterocyte.

Indeed, butyrate is foremost an energy source for the colonocyte and would be predicted to offset the effects of metabolic stress. Increasing the extracellular glucose concentration inhibited *E. coli* transcytosis (but not the drop in TER) across DNP-treated epithelia; an event not seen with mannitol or 3-OMG indicating that neither osmotic load nor activation of a glucose transporter alone mediated the effect of glucose. Thus, the diminution of *E. coli* transcytosis across metabolically stressed T84 epithelia by butyrate could be a combination of blockade of NF-κB signaling and provision of an energy source. Butyrate is neither the only nor the most abundant

35 Sasaki *et al.*, 2007
36 Rook & Stannford, 1998
37 Venkatraman *et al.*, 2003; Sauer *et al.*, 2007
38 Segain *et al.*, 2000; Yin *et al.*, 2001
39 Lewis *et al.*, 2008
40 Mariadason *et al.*, 1997

EFFICIENT CONTROL OF EPITHELIAL PERMEABILITY IS OF PARAMOUNT IMPORTANCE TO HEALTH

BUTYRATE ANTAGONIZES THE EFFECT OF METABOLIC STRESS IN COLONOCYTES AND ABLATES BACTERIAL TRANS-LOCATION ARISING AS A CONSEQUENCE OF PERTURBED MITOCHONDRIAL FUNCTION

SCFA in the colon, where acetate is in greater abundance[41]. However, acetate was unable to prevent the increased bacterial translocation across DNP-treated T84 monolayers, which is compatible with its reduced ability to block NF-κB activation[42] and lack of metabolism in colonocytes[43].

Butyrate enemas or prebiotics (i.e. oligosaccharide substrates for bacterial fermentation and SCFA synthesis) have been successful in reducing colitis in rodent models [44] and success with similar strategies as maintenance therapies in IBD and other enteropathies has been reported[45]. However, data are scant in this area and other studies have equally convincingly shown no therapeutic value of butyrate enemas in ulcerative colitis[46]. Many variables could explain the discrepancies between these studies, such as, for example, the severity of the disease at the time of treatment. Nevertheless based on the analysis of epithelial permeability presented here, and those reported by others[47] we speculate that prebiotics would be of value as a prophylactic in patients with IBD, and perhaps particularly so at times of stress, when some patients can be at greater risk for relapse[48] and noting that epithelial mitochondrial abnormalities can occur following psychological stress[49].

In summary, butyrate antagonizes the effect of metabolic stress in the colonocyte and ablates bacterial translocation arising as a consequence of perturbed mitochondrial function in gut-derived polarized epithelia. These novel findings underscore the importance of fermentable carbohydrates and a normal gut microflora in the maintenance of epithelial barrier function as part of the host innate defense strategy.

ACKNOWLEDGEMENTS

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41 Cook & Sellin, 1998
42 Tedelind *et al.*, 2007
43 Cook & Sellin, 1998; Miller, 2004
44 Hoentjen *et al.*, 2005; Looijer-van Langen & Dieleman, 2009; Mariadason *et al.*, 1999; Venkatraman *et al.*, 2003
45 Scheppach *et al.*, 1992; Okamoto *et al.*, 2000; Kiely *et al.*, 2001
46 Steinhart *et al.*, 1996
47 Peng *et al.*, 2007; Venkatraman *et al.*, 2003; Mariadason *et al.*, 1999; Looijer-van Langen & Dieleman, 2009
48 Bernstein *et al.*, 2006
49 Soderholm *et al.*, 2002

4

Modification of intestinal flora with multispecies probiotics reduces bacterial translocation and improves clinical course in a rat model of acute pancreatitis.

Probiotics prevent bacterial translocation

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ABSTRACT

BACKGROUND

Infection of pancreatic necrosis by gut bacteria is a major cause of morbidity and mortality in patients with severe acute pancreatitis. Use of prophylactic antibiotics remains controversial. Aim of this experiment was to assess if modification of intestinal flora with specifically designed multispecies probiotics[1] reduces bacterial translocation or improves outcome in a rat model of acute pancreatitis.

METHODS

Male Sprague-Dawley rats were allocated into three groups: 1) controls (sham operated, no treatment), 2) pancreatitis and placebo or 3) pancreatitis and probiotics. Acute pancreatitis was induced by intraductal glycodeoxycholate and intravenous cerulein infusion. Daily probiotics or placebo were administered intragastrically, five days before until seven days after induction of pancreatitis. Tissue and fluid samples were collected for microbiological and quantitative real-time PCR analysis of bacterial translocation.

1 (*Ecologic® 641*)

RESULTS

Probiotics reduced duodenal bacterial overgrowth of potential pathogens[2], resulting in reduced bacterial translocation to extra-intestinal sites, including the pancreas[3]. Accordingly, health scores were better and late phase mortality was reduced[4].

CONCLUSIONS

This experiment supports the hypothesis that modification of intestinal flora with multispecies probiotics results in reduced bacterial translocation, morbidity and mortality in the course of experimental acute pancreatitis.

2 Log_{10} colony forming units (CFU)/g 5.0 ± 0.7 (placebo) *vs.* 3.5 ± 0.3 CFU/g (probiotics), $P < 0.05$
3 5.4 ± 1.0 CFU/g (placebo) *vs.* 3.1 ± 0.5 CFU/g (probiotics), $P < 0.05$
4 27% (4/15, placebo) *vs.* 0% (0/13, probiotics) respectively, $P < 0.05$

INTRODUCTION

Severe acute pancreatitis follows a biphasic clinical course. The early pro-inflammatory phase is associated with systemic inflammatory response syndrome (SIRS), (multiple) organ damage and early mortality (<1 week). The late phase is characterized by infectious complications following bacterial translocation of intestinal bacteria and late mortality (>3 weeks)[5]. Infectious complications are frequently the cause of mortality in acute pancreatitis patients[6].

The use of prophylactic antibiotics to prevent infectious complications remains a topic of debate. A recent meta-analysis of six randomized controlled trials concluded that prophylactic antibiotics do not prevent infection of pancreatic necrosis or mortality in severe acute pancreatitis. Furthermore, increased concerns regarding the widespread use of prophylactic antibiotics associated complications (*i.e.* fungal infections or antibiotics resistance) have been reported[7]. Prophylactic probiotics have been suggested as an alternative to the use of prophylactic antibiotics[8]. Beneficial effects of prophylactic probiotics for acute pancreatitis have been reported in animal experiments and clinical trails[9]. Most studies on prophylactic probiotics however, have focused on a single probiotic strain for a variety of medical disorders. A recent report however, has advocated the use of specifically selected multiple probiotic strains[10]. For optimal results, probiotic strains should be selected to target known pathophysiological aspects of the disorder addressed[11].

Experimental and clinical studies have greatly increased the knowledge of the pathophysiology of bacterial translocation during acute pancreatitis. Three major steps in the sequence of bacterial translocation have been identified: 1) small bowel bacterial overgrowth, 2) mucosal barrier failure and 3) pro-inflammatory responses[12]. These three phenomena occur early after the start of acute pancreatitis and cumulate into bacterial translocation and infectious complications. Based on these considerations, we have designed a mixture of six probiotic strains, especially selected to reduce small bowel bacterial overgrowth and reduce pro-inflammatory immune responses[13].

It is unknown if modification of the intestinal flora with such a multispecies probiotic mixture reduces bacterial translocation and consequently, alter the course of disease. Therefore, the aim of the present study was to assess if modification of intestinal flora by a specifically designed, multispecies probiotic mixture changes disease course using a well established rat model of acute pancreatitis.

5 Buchler *et al.*, 2000; Beger *et al.*, 2005
6 Widdison & Karanjia, 1993; Isenmann *et al.*, 2001
7 Grewe *et al.*, 1999; De Waele *et al.*, 2004
8 Besselink *et al.*, 2005
9 Olah *et al.*, 2002; Mangiante *et al.*, 2001
10 Timmerman *et al.*, 2004
11 Isolauri *et al.*, 2002
12 Van Felius *et al.*, 2003; Berg, 1999
13 Timmerman *et al.* Manuscript submitted for publication

ANTIBIOTICS DO NOT PREVENT INFECTION OF PANCREATIC NECROSIS OR MORTALITY

THE AIM WAS TO ASSESS IF MODIFICATION OF INTESTINAL FLORA BY A SPECIFICALLY DESIGNED, MULTISPECIES PROBIOTIC MIXTURE CHANGES DISEASE COURSE

MATERIALS AND METHODS

ANIMALS

Male specific pathogen-free Sprague-Dawley rats, 250-350 grams (Harlan, Horst, the Netherlands) were kept under constant housing conditions (temperature (22°C), relative humidity (60%) and a 12-hour light/dark cycle) and had free access to water and food (RMH 1110, Hope Farms, Woerden, the Netherlands) throughout the experiment. Rats were allowed to adjust to these conditions for one week prior to surgery. The experimental design, shown in Figure 1, was approved by the institutional animal care committee of the University Medical Center, Utrecht, the Netherlands. Rats were randomized (1: 2: 2 relative group size computer generated randomization for the respective groups) between three experimental groups: 1) control animals (gastric cannula, sham pancreatitis, no treatment), 2) acute pancreatitis and placebo, and 3) acute pancreatitis and probiotics. In total, 10 rats were included in the control group, 21 rats in the placebo group, and 17 rats in the group given daily probiotics.

PROBIOTICS AND PLACEBO

The study product (*Ecologic® 641*, Winclove BioIndustries, Amsterdam, the Netherlands) consisted of viable and freeze-dried probiotic strains; 4 lactobacilli: *Lactobacillus acidophilus* (W70), *Lactobacillus casei* (W56), *Lactobacillus salivarius* (W24), *Lactococcus lactis* (W58), and two bifidobacteria: *Bifidobacterium bifidum* (W23) and *Bifidobacterium infantis* (W52). The placebo product consisted of carrier substance only (corn-starch). Probiotics and placebo were packed in identical sachets and coded by the producer to guarantee blinding during the experiment. Directly before administration of the doses, the products were reconstituted in sterile water, for 15 minutes at 37°C. Single probiotics dose volume of 1.0 ml contained a total of 5 x 10^9 CFU bacteria. Probiotics or placebo were administered intragastrically through a permanent gastric cannula once daily, starting five days prior to induction of acute pancreatitis, and twice daily for six days after induction of acute pancreatitis.

SURGICAL PROCEDURES

All surgical procedures were performed on a heated operating table under general anaesthesia using a combination of 2% Isoflurane gas (flow: 0.5 l/min O_2, 1.5 l/m air) through a snout-mask and intramuscular 0.3 ml 10% buprenonorphine (Temgesic, Reckitt Beckiser Healthcare Ltd., Hull, UK). All surgical procedures were performed with sterile instruments under strict aseptic conditions. Throughout the experiment random control swabs of the abdomen remained negative on bacterial cultures, ensuring external contamination did not occur.

GASTRIC CANNULATION

At the start of the experiment, a permanent gastric cannula was fitted in all rats. Under general anesthesia, a 20 cm silicone cannula (outer diameter 1.65 mm, inner diameter 0.76 mm, Rubber, Amsterdam, the Netherlands) was tunneled

subcutaneously from the abdominal wall to the back, penetrating the skin between the scapulae. A 1.5 cm midline laparotomy was made to insert the gastric end of the cannula into the stomach through a puncture within a purse-string suture on the greater curvature. The cannula was securely fixed and the abdomen was closed in two layers. The dorsal end of the cannula was kept in place between the scapulae using a rodent infusion jacket (Uno Zevenaar BV, Zevenaar, the Netherlands). Animals in the probiotics and placebo groups were allowed to recover for three days, prior to the start of daily probiotics or placebo administrations.

INDUCTION OF ACUTE PANCREATITIS

Five days after starting daily administration of placebo or probiotics, acute pancreatitis was induced using the internationally well accepted model described by Schmidt *et al. (1992).* Briefly, during midline relaparotomy the papilla of Vater was cannulated transduodenally using a 24G Abbocath®-T i.v. infusion cannula (Abbott, Sligo, Republic of Ireland) (fig 2). Before pressure monitored infusion (MMS, Enschede, the Netherlands) of 0.5 ml sterilized glycodeoxycholic acid in glycylglycine-NaOH-buffered solution (10 mmol/l, pH 8.0, 37°C, chemicals obtained from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), the common bile duct was clamped and bile and pancreatic fluid were allowed to drain through the cannula. No animals needed to be excluded for infusion pressures exceeding 35 mmHg. Directly after infusion, hepato-duodenal bile flow was restored by removal of the clamp. The puncture hole in the duodenum was carefully closed using an 8.0 polyprolene serosal suture (fig 2) After closure of the abdomen in two layers, the right jugular vein was cannulated for continuous postoperative intravenous infusion of cerulein (5µg/kg/hr, for six hours). The jugular vein cannula was fixed to the rodent infusion jacket and attached to a swivel system to provide unrestricted mobility of the rat during infusion. During the sham procedure in the control rats, the papilla of Vater was cannulated, the common bile duct was clamped, but no glycodeoxycholic acid was infused. A jugular vein cannula was fitted for six hours of intravenous saline infusion. After acute pancreatitis induction or sham procedure, pain relief was provided for 48 hours by subcutaneous injections of 0.3 ml 10% buprenonorphine twice daily.

The clinical response of the rats after induction of acute pancreatitis was assessed using a 0-6 points scoring system: *Grooming*: normal = 2 points, decreased = 1 point, none = 0 points. *Mobility*: normal = 2 points, decreased = 1 point, immobile = 0 points. *Pain posture*: none = 2 points, arching (convex back and retraction of abdomen from floor) = 1 point, stretching (whole body is stretched out on floor, spine is straight and horizontal) = 0 points. Aspects of this scoring system are well recognized behavioral parameters expressing health or morbidity (including abdominal / visceral pain)[14]. According to Dutch animal welfare laws and local protocols of the animal ethics committee, daily assessments of these aspects are mandatory to monitor animal welfare throughout the experimental protocol. Indeed, two rats in the placebo group demonstrating signs of severe suffering and poor clinical prognosis (low health scores) were terminated on day six and added to the Kaplan-Meier statistic the same day.

14 Stam *et al.*, 2004; Houghton *et al.*, 1997

FIGURE 1

Experimental design

Eight days prior to induction of acute pancreatitis, a permanent gastric cannula was fitted. Probiotics or placebo were administered intragastrically through a permanent gastric cannula once daily, starting 5 days prior to induction of acute pancreatitis, and twice daily from days 1-7 after induction of acute pancreatitis. Seven days after induction of pancreatitis, surviving rats were anesthetized to allow sterile removal of organ and blood samples. Control animals did not receive administrations through the gastric cannula and underwent a sham pancreatitis procedure only.

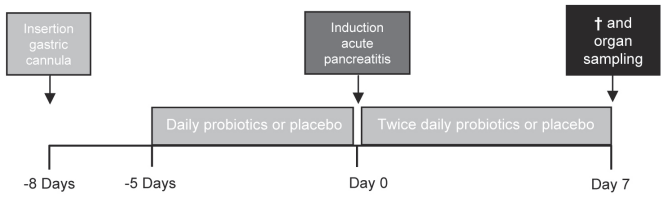
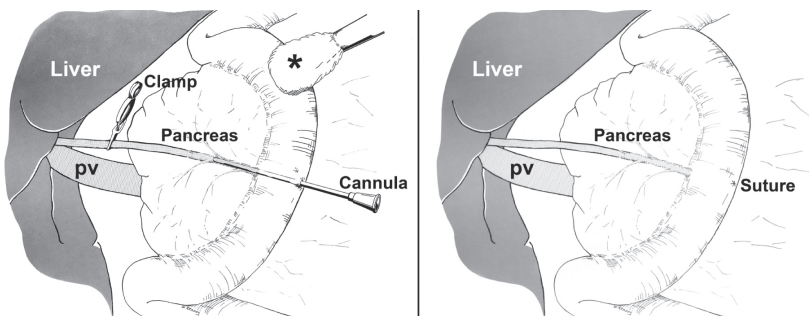


FIGURE 2

Schematic representation of the bile infusion model according to Schmidt *et al.* (1992)

The duodenum is held aside with a cotton wool stick (*), pv: portal vein. After clamping of the common bile duct the papilla of Vater is cannulated transduodenally for bile salt infusion (left panel). Directly after infusion, hepato-duodenal bile flow was restored by removal of the clamp. The puncture hole in the duodenum was sutured (right panel).



COLLECTION OF FLUID AND TISSUE SAMPLES

On day seven, surviving rats were anesthetized to allow sterile removal of organ and fluid samples. To avoid cross-contamination, samples were taken under strict aseptic conditions in the following order: peritoneal fluid, blood (inferior vena cava), mesenteric lymph nodes (MLN), liver, spleen, pancreas and duodenum. After sample collection, rats were euthanized by blood loss. Samples were collected for microbiological analysis and portion of each sample and a segment of the ileum was snap frozen in liquid nitrogen and stored at -80°C for future analysis. Another portion of pancreatic samples was analyzed histopathologically, using standard hematoxilin and eosin (H&E) staining. Histopathological severity of acute pancreatitis was assessed based on the acute pancreatitis scoring system as previously described[15].

CULTURE-BASED MICROBIOLOGICAL ANALYSIS FOR BACTERIAL IDENTIFICATION AND QUANTIFICATION

All organ samples were weighed and processed immediately for quantitative and qualitative cultures of aerobic and anaerobic organisms. All organs were homogenized in cysteine broth with a sterile blender and cultured in 10-fold dilution series. The samples were cultured on bloodagar, MacConkey-agar (for Gram-negative strains), Columbia Colistin Nalidixic Acid (CNA) agar (for staphylococci and streptococci), Man-Rogosa-Sharpe-agar (for lactobacilli) and Schaedler agar (for facultative anaerobic bacteria). The microorganisms were identified using standard microbiological techniques. For analysis of organ samples, cultured bacteria were subdivided in three groups: gram-positive cocci (GPC), gram-positive rods (GPR) and gram-negative rods / anaerobes (GNR+anear). Also, Hemolytic *Streptococcus* group B, *Enterococcus spp.*, *Staphylococcus aureus,* and Enterobacteriaceae such as *Escherichia coli*, *Proteus mirabilis* and *Morganella morganii* were categorized as potential pathogens. Bacterial counts are expressed as Log_{10} colony forming units per gram tissue (CFU/g) ± standard error of the mean. Threshold detection level of bacterial growth was $>10^2$ CFU/g.

DNA ISOLATION AND REAL-TIME PCR ASSAY FOR TOTAL BACTERIAL QUANTIFICATION

DNA was isolated from mesenteric lymph nodes and pancreas homogenates using Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad, CA, USA) as previously described[16]. Subsequently, total bacterial quantification was performed employing 16S rRNA gene-targeted primers, 968F (5'- AAC GCG AAG AAC CTT AC -3') and R1401 (5'-CGG TGT GTA CAA GAC CC-3'). Real-time PCR was done on an iCycler IQ real-time detection system coupled to the iCycler optical system interface software version 2.3 (Bio-Rad, Veenendaal, the Netherlands). The reaction mixture (25µl) consisted of 12.5 µl of IQ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer set, and 5 µl of the template DNA. The PCR conditions for total bacterial quantification were: 94 °C for 5 min, and 35 cycles of 94 °C for

15 Konstantinov *et al.*, 2004
16 van Minnen *et al.*, 2006

30 sec, 56 °C for 20 sec, 68 °C for 40 sec. Serially diluted genomic DNA of selected bacterial isolates was used as real-time PCR control for total bacteria quantification. PCR bacterial counts are expressed as Log_{10} cells per gram tissue (cells/g) ± standard error of the mean.

STATISTICAL ANALYSIS

Survival rates were analyzed with Kaplan-Meier analysis. Health scores and incidence of positive bacterial cultures were compared between groups using the non-parametric Mann-Whitney U test. Bacterial counts (cultures) and cell counts (PCR) were analyzed using *t*-tests for relevant subgroups (SPSS 12.0 statistical software, SPSS Benelux, Gorinchem, the Netherlands). Spearman's rank correlation coefficients were computed for linear correlation analyses. Results are presented as mean ± standard error of the mean. Culture results are presented as mean Log_{10} colony forming units (CFU)/gram tissue and quantitative real-time PCR results as Log_{10} cells/gram tissue. Statistical significance was accepted when 2-tailed *P*-values were below 0.05.

RESULTS

MORBIDITY AND MORTALITY

After the start of daily placebo or probiotic administrations, physical behavior of all rats remained normal, resulting in maximal health scores from day -5 until day 0. The clinical response of the rats after induction of experimental pancreatitis followed a biphasic course. During the first 72 hours, the animals exhibited decreased grooming or motility and to some extent behavior associated with pain, despite analgesic administration during the first 48 hours. From days 3 to 5, surviving animals apparently recovered, evidenced by near to normal physical behavior. After day 5, rats deteriorated, resulting in a second decrease of health-scores. Throughout days 1 to 7, median health scores of surviving rats were higher for rats in the probiotics group compared to those in the placebo group, with significant contrasts on days 1, 2 and 3[17]. An interpretation of the biphasic course of acute pancreatitis is visualized by the curves superimposed on the median health scores shown in figure 3.

In the pancreatitis groups, histological examination of the pancreatic samples revealed late sequelae of severe necrotizing acute pancreatitis (fig 4). The extent of necrosis, hemorrhage, inflammatory infiltrate or fibrosis was comparable for the probiotics and placebo groups, suggesting rats of both pancreatitis groups were subject to acute pancreatitis of equal severity.

Overall mortality due to acute pancreatitis was 37% (14/38). Mortality in the probiotics group was 24% (4/17) and 48% (10/21) in the placebo group ($P = 0.16$). Mortality within the first 24 and 48 hours was comparable between both groups[18]. However, late mortality (≥48 hours) did not occur in the probiotics group, resulting in a significant reduction of mortality compared to the placebo group[19]. The Kaplan-Meier survival curve for both pancreatitis groups is shown in figure 5. Rats that died before the scheduled 7 days after induction of acute pancreatitis were not analyzed further, leaving 34 rats for bacteriological analysis[20].

DUODENAL BACTERIAL OVERGROWTH AND BACTERIAL TRANSLOCATION

Total bacterial counts and numbers of lactobacilli in the duodenum were not significantly affected by induction of acute pancreatitis or administration of probiotics (fig 6). On the other hand, acute pancreatitis caused a significant increase in total counts of potential pathogens in the duodenum[21], compared to sham operated controls[22]. Most interestingly, probiotics prevented this duodenal bacterial overgrowth

17 median = 5 (range 3 – 6) *vs.* 4 (1 – 6), $P = 0.020$; 5 (4 – 6) *vs.* 4.5 (2 – 6), $P = 0.034$ and 6 (3 – 6) *vs.* 3 (1 – 5), $P < 0.001$, respectively

18 <24 hours: 12% (2/17) *vs.* 14% (3/21), < 48 hours: 24% (4/17) *vs.* 29% (6/21) for probiotics and placebo groups, respectively

19 ≥48 hours: 0% (0/13) *vs.* 27% (4/15) respectively, $P = 0.049$

20 controls: n = 10, placebo: n = 11, probiotics: n = 13

21 Hemolytic *Streptococcus* group B, *Enterococcus spp.*, *Staphylococcus aureus*, and Enterobacteriaceae such as *Escherichia coli*, *Proteus mirabilis* and *Morganella morganii*

22 5.0 ± 0.7 CFU/g *vs.* 2.9 ± 0.2 CFU/g, $P = 0.010$

of potential pathogens[23]. Most predominant effects of probiotics on duodenal flora were the reduction of enterococci and *E. coli*[24] (fig 6).

In rats of the placebo group, duodenal bacterial overgrowth correlated positively and significantly with bacterial translocation to the pancreas[25] (fig 7A). Strikingly, no correlation between duodenal bacterial overgrowth and infection of pancreatic necrosis was present in the probiotics group[26] (fig 7B).

MULTISPECIES PROBIOTICS REDUCE BACTERIAL TRANSLOCATION

Gram-negative rods / anaerobes were cultured less frequently in blood of rats of the probiotics group[27]. Induction of acute pancreatitis resulted in significant bacterial translocation to the mesenteric lymph nodes, spleen, liver and pancreas (fig 8 A-E). Rats in the probiotics group demonstrated significantly lower counts of microorganisms cultured in spleen, liver and pancreas compared to rats of the placebo group[28] (fig 8A). Bacterial counts of gram-positive cocci, gram-positive rods and gram-negative rods / anaerobes in the various abdominal organs are shown in figures 8B-E. Absolute bacterial counts were lower in all tissues of the probiotics group compared to placebo, and reached significance for gram-positive cocci in the spleen and for gram-negative rods / anaerobes in the spleen and liver[29] (fig 8C-D).

Escherichia coli and *Enterococcus* spp. were the most predominant bacteria found in tissues of rats with acute pancreatitis. Administration of probiotics resulted in significantly reduced bacterial growth of both *E. coli* and enterococci in the mesenteric lymph nodes[30]. Pancreatic counts of *E. coli* and enterococci were numerically reduced by probiotics, but failed to reach significant differences[31].

In line with the data obtained by bacterial culture, results of quantitative real-time PCR demonstrate lower bacterial translocation in the mesenteric lymph nodes and pancreas of rats in the probiotics group[32] (fig 9).

23 3.5 ± 0.3 CFU/g *vs.* 5.0 ± 0.7 CFU/g, for probiotics *vs.* placebo, respectively, $P = 0.048$

24 1.9 ± 0.2 CFU/g *vs.* 2.8 ± 0.6 CFU/g, $P = 0.159$ and 2.0 ± 0.2 CFU/g *vs.* 3.4 ± 0.8 CFU/g, $P = 0.032$, for probiotics *vs.* placebo, respectively

25 $R^2 = 0.80$, $P = 0.001$

26 $R^2 = 0.02$, $P = 0.829$

27 0/13 *vs.* 4/11, $P < 0.05$

28 2.4 ± 0.3 CFU/g *vs.* 3.7 ± 0.5 CFU/g , $P = 0.039$; 2.8 ± 0.8 CFU/g *vs.* 4.6 ± 0.8 CFU/g, $P = 0.049$; 3.1 ± 0.5 CFU/g *vs.* 5.4 ± 1.0 CFU/g, $P = 0.042$; respectively

29 2.3 ± 0.3 CFU/g *vs.* 3.5 ± 0.5 CFU/g, $P = 0.026$; 2.0 ± 0.1 CFU/g *vs.* 3.0 ± 0.5 CFU/g, $P = 0.032$; 2.0 ± 0.1 CFU/g *vs.* 3.1 ± 0.6 CFU/g, $P = 0.022$, respectively

30 1.9 ± 0.1 CFU/g *vs.* 2.6 ± 0.4 CFU/g, $P = 0.045$; 1.8 ± 0.05 CFU/g *vs.* 2.5 ± 0.3 CFU/g , $P = 0.015$, respectively

31 1.7 ± 0.01 CFU/g *vs.* 3.0 ± 0.7 CFU/g, $P = 0.067$; 1.7 ± 0.01 CFU/g *vs.* 3.0 ± 0.7 CFU/g, $P = 0.060$, respectively

32 5.9 ± 0.4 cells/g *vs.*7.0 ± 0.1 cells/g , $P = 0.043$, and 6.7 ± 0.3 cells/g *vs.* 7.7 ± 0.3 cells/g, $P = 0.013$, respectively

FIGURE 3

Median health scores after induction of acute pancreatitis

Median health scores after induction of acute pancreatitis, of placebo rats (▫) and rats of the probiotics group (•). Bars represent 25% - 75% interquartile range. Median health scores were improved by probiotics throughout days 1 to 7, with significant differences on days 1, 2 and 3 (* $P < 0.05$). Health scores of all rats were invariably 6 (maximum score) from day -5 until induction of acute pancreatitis (data not shown). The solid (probiotics) and dashed (placebo) lines are fitted to demonstrate an interpretation of the biphasic course of acute pancreatitis.

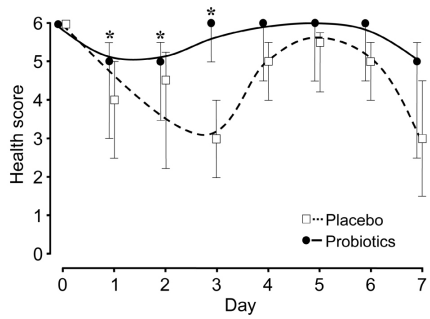


FIGURE 4

Induction of acute pancreatitis results in extensive pancreatic injury

(A) Normal pancreatic histology of control rats. (B) Histopathological sequelae of acute pancreatitis including destruction of acinar structures, fibrosis and a massive inflammatory infiltrate, 7 days after induction of acute pancreatitis (H&E staining, 100x).

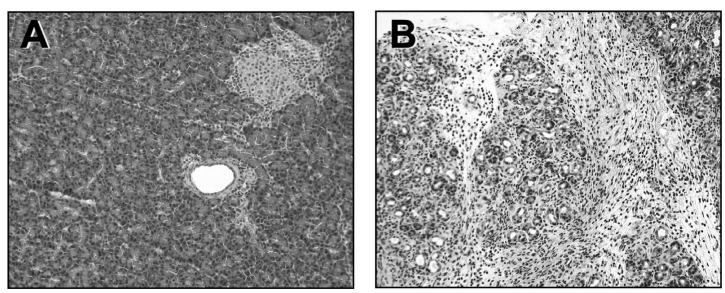


FIGURE 5

Probiotic pretreatment prevents late mortality

Kaplan-Meier survival plot of placebo (dashed line) and probiotics groups (solid line). Overall mortality placebo *vs.* probiotics: $P = 0.16$. Late mortality (≥ 2 days): $P < 0.05$

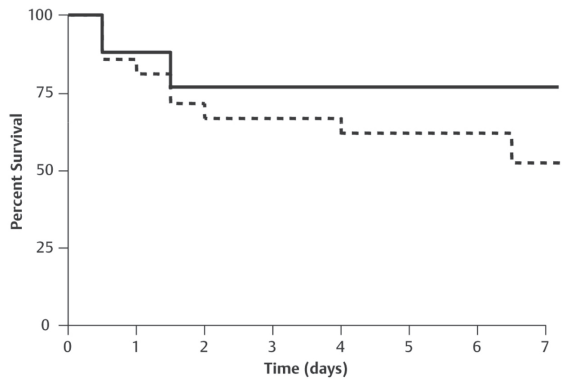


FIGURE 6

Pretreatment with probiotics prevents acute pancreatitis-induced bacterial overgrowth

Total duodenal bacterial counts and counts of lactobacilli, [1]potential pathogens (Hemolytic *Streptococcus* group B, *Enterococcus* spp., *Staphylococcus aureus*, and Enterobacteriaceae such as *Escherichia coli*, *Proteus mirabilis* and *Morganella morganii*), *E. coli*, and enterococci. Acute pancreatitis resulted in bacterial overgrowth of potential pathogens († Controls *vs.* placebo: $P < 0.05$). Probiotics (black bars) reduced bacterial counts of potential pathogens, mainly *E. coli*, compared to placebo (grey bars). (* Placebo *vs.* probiotics: $P < 0.05$).

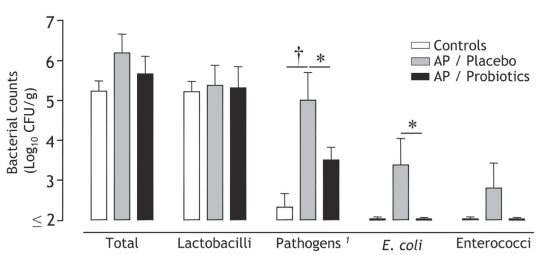


FIGURE 7

Correlations between duodenal bacterial counts and bacterial counts in the pancreas

Correlation between duodenal bacterial counts and bacterial counts in the pancreas for rats in the placebo (panel A) and probiotics (panel B) group. In rats of the placebo group, there is a significant correlation between duodenal and pancreatic bacterial counts ($P < 0.001$), whereas in the probiotics treated group there is not ($P > 0.05$).

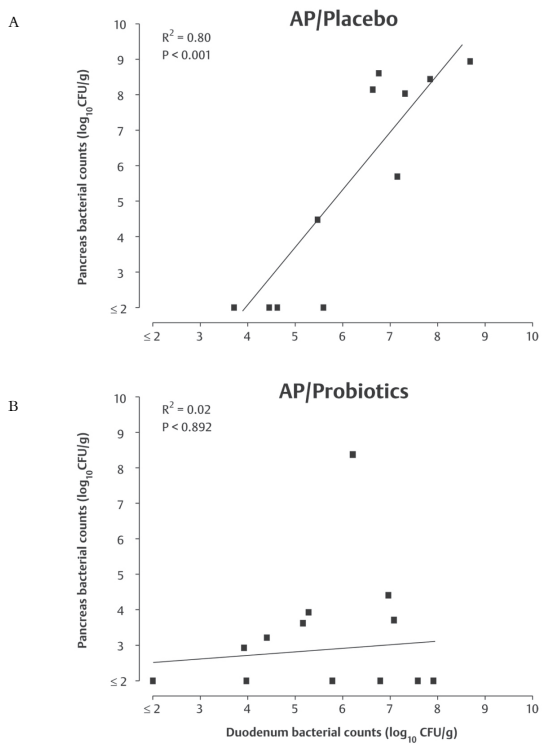
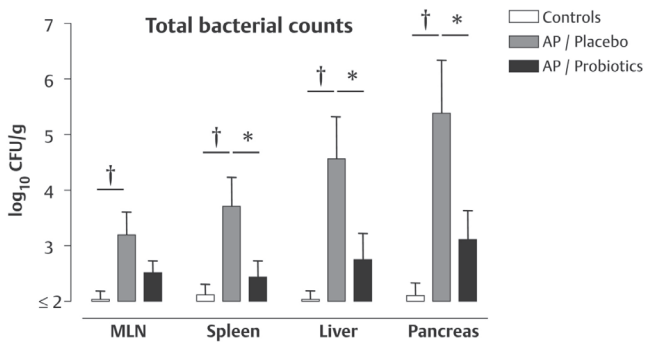


FIGURE 8

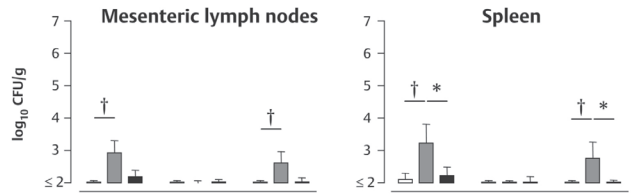
Probiotic pretreatment reduced acute pancreatitis-induced bacterial translocation

(A) Total bacterial counts in mesenteric lymph nodes (MLN), spleen, liver and pancreas in control rats (white bars), placebo rats (grey bars) and rats of the probiotics group (black bars). (B-E) Bacterial counts of gram-positive cocci (GPC), gram-positive rods (GPR) and gram-negative rods/anaerobes (GNR+anaer) in mesenteric lymph nodes (MLN; B), spleen (C), liver (D) and pancreas (E). * $P < 0.05$: placebo vs. probiotics, † $P < 0.05$ controls vs. placebo.

A



B / C



D / E

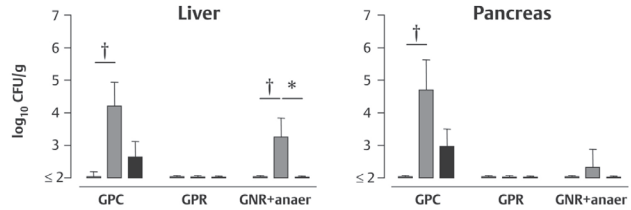
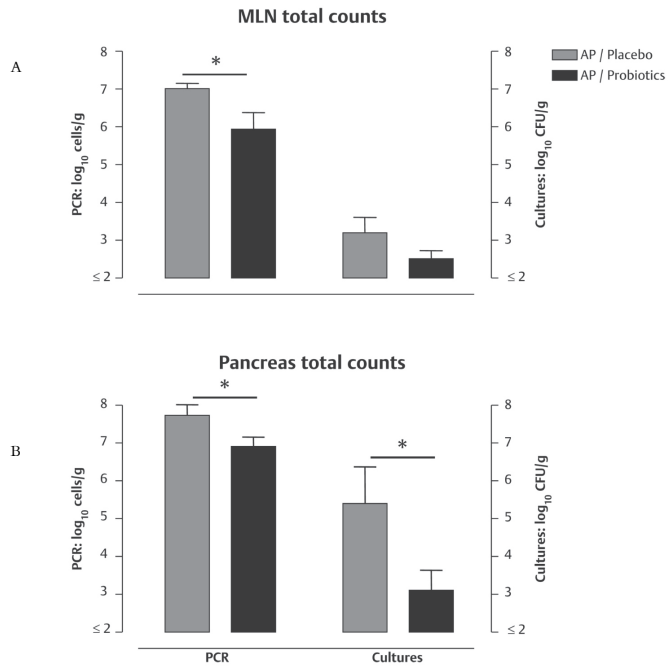


FIGURE 9

Quantification of total bacterial load by real-time PCR in placebo rats (grey bars) and rats of the probiotics group (black bars) in mesenteric lymph nodes (A) and pancreas (B). Data of microbiological cultures is shown for comparison and are also presented in figure 8. * $P < 0.05$



DISCUSSION

This is the first study to assess the potential of a specifically designed multispecies probiotic mixture to reduce bacterial translocation during acute pancreatitis. In this paper we demonstrate that modification of intestinal flora with this probiotic mixture alters the course of experimental acute pancreatitis. Administration of the selected probiotic mixture resulted in *1)* reduced duodenal overgrowth of pathogens, such as *E. coli*, *2)* reduced bacterial translocation to distant organs, including the pancreas, *3)* improved clinical course and *4)* reduced late mortality.

Results confirm our earlier reports that small bowel bacterial overgrowth during acute pancreatitis correlates with infection of pancreatic necrosis[33]. Daily administration of probiotics did not increase the duodenal total count of lactobacilli. On the other hand, functionality of the administered probiotics is demonstrated by the significantly reduced numbers of potential pathogens in the duodenum, particularly *E. coli*. Reduction of acute pancreatitis induced pathogen overgrowth in the small bowel resulted in reduced bacterial infection of pancreatic necrosis in rats of the probiotics group. Reduced presence of luminal pathogens may have had favorable effects on mucosal barrier function of the proximal small bowel, reducing bacterial translocation. However, effects of the selected probiotic mixture on mucosal barrier function remains to be investigated in additional experimental studies.

Microbiological cultures demonstrated a significant decrease of bacterial growth in spleen, liver and pancreas in rats of the probiotics group. Confirming these results, quantitative real-time PCR detection of bacterial DNA revealed a significant decrease of bacterial translocation to mesenteric lymph nodes and pancreas. Bacterial DNA is stable, and PCR based methods are highly sensitive and specific to detect minimal amounts of bacterial DNA in serum of patients with acute pancreatitis[34]. This method detects not only viable, but also non-viable translocated bacteria, probably killed by the host immune system. Therefore, total bacterial load estimated by real-time PCR is higher than counts of viable microorganisms only. Moreover, average reduction of pancreatic bacterial load by probiotic prophylaxis was greater when analyzed by culture (>2 Log) then by PCR (<1 Log). Thus, reduction of viable bacteria cultured from pancreatic necrosis cannot be completely explained by an absolute reduction of bacterial translocation, as indicated by quantitative real-time PCR. It could be suggested that probiotic prophylaxis renders the immune system more capable to kill translocated bacteria in distant organs. In follow-up studies we are currently addressing effects of enteral probiotics on a wide panel of plasma cytokines to assess immune modulatory potential in the early and late phase of experimental acute pancreatitis.

Rats in the probiotics group showed less stress- or pain-associated behavior, demonstrated by objective improvement in the clinical course of experimental pancreatitis. Albeit a biphasic course in clinical presentation could still be identified in rats of the probiotics group, health scores were clearly improved compared to placebo rats. Moreover, probiotic prophylaxis numerically reduced overall mortality

33 Van Felius *et al.*, 2003
34 de Madaria *et al.*, 2005

of acute pancreatitis, and a significant reduction was observed in late phase mortality. In humans, infectious complications are held accountable for late phase mortality[35]. In line with these reports, reduced infectious complications in probiotic treated rats were associated with reduced late phase mortality in the present experiment. Probiotics did not affect histological severity, assessed seven days after induction of acute pancreatitis. Early phase histological changes were not assessed.

The experimental design of the present study aimed to assess the effect of gut flora modulation by probiotics on the course of experimental acute pancreatitis, using bacterial translocation as a major outcome parameter. Therefore, rats were pretreated with the selected probiotics or placebo. In experimental acute pancreatitis, timing of the start of treatment remains a challenging issue. The course of acute pancreatitis in rats is approximately 3 to 6 times faster than in man[36]. This leaves only a small treatment-window between onset of disease and occurrence of complications, potentially leading to false negative results if treatment is started after induction of pancreatitis. For this reason treatment started before induction of acute pancreatitis in many other studies[37]. Also, for probiotics in particular, assessment of their efficacy by pretreatment is an accepted experimental method to provide proof of principle[38]. We emphasize that results of the present experiment do not necessarily reflect potential results if treatment is started after the onset of acute pancreatitis in general, and potential clinical success or validity in particular.

For a prophylactic strategy to be effective, it should intervene with the pathophysiology of bacterial translocation during acute pancreatitis as early as possible. The exact pathophysiology of bacterial translocation, infection of pancreatic necrosis and the ensuing systemic effects is still not fully understood. Yet, the sequence of some major pathophysiological aspects has been clarified. Early after the onset of acute pancreatitis, neurohormonal effects result in reduced small bowel motility[39]. This causes stasis of luminal contents and small bowel bacterial overgrowth with potential pathogens, including *E. coli* and *Enterococcus* species. The abundant presence of luminal pathogens forms a challenge for the mucosal barrier. Furthermore, pancreatitis associated reduced intestinal blood flow results in mucosal ischemia and reperfusion damage[40]. Luminal bacteria, normally held at bay by the mucosal barrier, now have opportunity to penetrate into the intestinal epithelium. Local intestinal inflammation follows, further compromising mucosal barrier function. Pancreatitis and ensuing intestinal inflammation both contribute to a systemic pro-inflammatory response (Systemic Inflammatory Response Syndrome, SIRS), with damaging effects on distant organs[41]. If the systemic response is severe, multiple organ dysfunction syndrome (MODS) might follow[42]. If the patient survives the early phase, counter regulatory immunological pathways releasing anti-inflammatory cytokines result in a refractory state characterised by immunosuppression. Persistent immunosuppression will render the patient liable for infection of pancreatic necrosis. MODS caused

35 Widdison & Karanjia, 1993
36 Foitzik *et al.*, 2000
37 Mithofer *et al.*, 1996
38 Zareie *et al.*, 2006
39 Van Felius *et al.*, 2003
40 Inoue *et al.*, 2003
41 McKay & Imrie, 2004; Tran *et al.*, 1993
42 Bhatia, 2005

TIMING OF THE START OF TREATMENT REMAINS A CHALLENGING ISSUE

RESULTS
OF THE PRESENT
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REFLECT
POTENTIAL
RESULTS IF
TREATMENT IS
STARTED AFTER
THE ONSET
OF ACUTE
PANCREATITIS

by infectious complications is considered accountable for so-called late mortality or "late septic death"[43].

With this pathophysiology of local and systemic events during severe acute pancreatitis in mind, six probiotic strains were selected for this study. Selection of strains was based on their *in vitro* antibacterial and immunomodulatory properties[44]. *Lactobacillus acidophilus* and *Lactobacillus salivarius* were selected for their ability to suppress growth of *E. coli* and enterococci. *Bifidobacterium infantis* also demonstrated antimicrobial effects. *Lactococcus lactis* and *Bifidobacterium bifidum* demonstrated immune-modulating properties, including decreasing pro-inflammatory and increasing anti-inflammatory immune-responses. Finally, *Lactobacillus casei* demonstrated both antimicrobial and immune-modulating properties.

In thorough reviews on the use of animal models of acute pancreatitis, the used model was preferred to examine pathophysiology of bacterial translocation and for testing treatment strategies. Resemblance to human acute pancreatitis with regard to bacteriological results, reaction to treatment, and disease course are the major advantages[45]. The transduodenal approach to the biliopancreatic duct for bile salt infusion is often suggested to be a major drawback of the model for its potential to introduce bacteria in pancreatic tissue. However, results of the control group in the present study once more confirm that transduodenal cannulation of the biliopancreatic duct does not result in bacterial contamination of any concern to study outcome. Because of its demonstrated value, the model has previously been applied in many experiments testing the value of antibiotics during acute pancreatitis[46].

Experimentally, prophylactic antibiotics reduced overgrowth of *E. coli* and enterococci in the small bowel, resulted in significantly reduced bacterial translocation to distant organs, including the pancreas, and reduced mortality[47]. Unfortunately, clinical results of prophylactic antibiotics were not as successful. In a recent placebo-controlled double blinded clinical trial, Isenmann *et al.* (2004) demonstrated that prophylactic antibiotics (ciprofloxacin/metronidazole) showed no effect on bacterial infection of pancreatic necrosis or clinical outcome. Furthermore, concerns on prophylactic use of broad-spectrum antibiotics have been expressed, including increased incidence of nosocomial infections with resistant bacteria or fungi[48]. In this context, specifically selected multispecies probiotics as presented in this study, may be a novel and potentially effective alternative. However, as was demonstrated by the contrast between experimental and clinical results of antibiotics in acute pancreatitis, the clinical value of specifically selected multispecies probiotics remains to be proven. For this reason, the Dutch Acute Pancreatitis Study Group embarked on a randomized double blind placebo-controlled multicenter trial on prophylactic multispecies probiotics in patients with predicted severe acute pancreatitis.

In summary; modification of intestinal flora with multispecies probiotics, especially designed to address pathophysiology of bacterial translocation, resulted in reduced small bowel bacterial overgrowth, bacterial translocation to distant organs and associated morbidity and late mortality in experimental acute pancreatitis.

43 Gloor *et al.*, 2001
44 Timmerman HM, *et al.*, manuscript in preparation
45 Foitzik *et al.*, 2000
46 Mithofer *et al.*, 1996; Gloor *et al.*, 2003
47 Beger *et al.*, 2005
48 Murray, 2000

5

Probiotics prevent intestinal barrier dysfunction in acute pancreatitis in rats via induction of ileal mucosal glutathione biosynthesis.

Probiotics maintain barrier

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ABSTRACT

BACKGROUND

During acute pancreatitis (AP), oxidative stress contributes to intestinal barrier failure. We studied actions of multispecies probiotics on barrier dysfunction and oxidative stress in experimental AP.

METHODS

Fifty-three male Spraque-Dawley rats were randomly allocated into five groups: 1) controls, non-operated, 2) sham-operated, 3) AP, 4) AP and probiotics and 5) AP and placebo. AP was induced by intraductal glycodeoxycholate infusion and intravenous cerulein (6h). Daily probiotics or placebo were administered intragastrically, starting five days prior to AP. After cerulein infusion, ileal mucosa was collected for measurements of *E. coli* K12 and ^{51}Cr-EDTA passage in Ussing chambers. Tight junction proteins were investigated by confocal immunofluorescence imaging. Ileal mucosal apoptosis, lipid peroxidation, and glutathione levels were determined and glutamate-cysteine-ligase activity and expression were quantified.

RESULTS

AP-induced barrier dysfunction was characterized by epithelial cell apoptosis and alterations of tight junction proteins (i.e. disruption of occludin and claudin-1 and up-regulation of claudin-2) and correlated with lipid peroxidation ($r>0.8$). Probiotic

pretreatment diminished the AP-induced increase in *E. coli* passage[1], ^{51}Cr-EDTA flux[2], apoptosis, lipid peroxidation[3], and prevented tight junction protein disruption. AP-induced decline in glutathione was not only prevented[4], but probiotics even increased mucosal glutathione compared with sham rats[5]. Glutamate-cysteine-ligase activity, which is rate-limiting in glutathione biosynthesis, was enhanced in probiotic pretreated animals[6] coinciding with an increase in mRNA expression of glutamate-cysteine-ligase catalytic (GCLc) and modifier (GCLm) subunits.

CONCLUSIONS

Probiotic pretreatment diminished AP-induced intestinal barrier dysfunction and prevented oxidative stress via mechanisms mainly involving mucosal glutathione biosynthesis.

1 probiotics 57.4 ± 33.5 *vs.* placebo 223.7 ± 93.7 a.u.; $P<0.001$
2 probiotics 16.7 ± 10.1 *vs.* placebo 32.1 ± 10.0 cm/s10^{-6}; $P<0.005$
3 probiotics 0.42 ± 0.13 *vs.* placebo 1.62 ± 0.53 pmol MDA/mg protein; $P<0.001$
4 probiotics 14.33 ± 1.47 *vs.* placebo 8.82 ± 1.30 nmol/mg protein, $P<0.001$
5 probiotics 14.33 ± 1.47 *vs.* placebo 10.70 ± 1.74 nmol/mg protein, $P<0.001$
6 probiotics 2.88 ± 1.21 *vs.* placebo 1.94 ± 0.55 nmol/min/mg protein; $P<0.05$

INTRODUCTION

Multi-organ-failure and systemic inflammatory response syndrome (SIRS) remain major causes of mortality at intensive care units[7]. There is compelling evidence for an important role of the gut in the origin and development of critical illness[8]. Gut barrier dysfunction can propagate a pathophysiological state leading to increased mortality. Deitch *et al.* (1994) demonstrated for example, that shock-induced intestinal hypoperfusion leads to release of reactive oxygen species (ROS) and oxidative stress resulting in barrier failure and release of pro-inflammatory mediators, enhancing a subsequent SIRS. Evidence suggests that ROS disrupt epithelial tight junctions (TJs) [9]leading to barrier dysfunction[10]. Furthermore, ROS cause epithelial cell apoptosis [11] contributing to mucosal barrier failure [12] and associated mortality [13]in experimental studies. Moreover, clinical evidence shows that increased intestinal apoptosis is a prominent event in patients who succumb from sepsis[14]. The mucosal barrier may be further compromised by overgrowth of enteric pathogens e.g. *Escherichia coli* [15] or by other opportunistic pathogens which switch on their virulence genes upon intestinal hypoxia[16], suggesting an important role for intestinal microbiota in gut-derived sepsis[17]. Taken together in critically ill patients, SIRS may be driven by an oxidative stress-induced disruption of the equilibrium of the otherwise symbiotic three-way partnership between intestinal microbiota, epithelium, and immune system.

Conversely, a moderate increase in intracellular ROS concentrations may paradoxically afford protection against oxidative stress *via* upregulation of oxidative defense mechanisms. Indeed, *de novo* synthesis of the most important endogenous antioxidant, glutathione (GSH) is found to be enhanced after low dose H_2O_2[18] and is also increased by other weak oxidative agents[19].

Severe acute pancreatitis (AP), which is characterized by intestinal barrier dysfunction and not seldom leading to SIRS, represents a clinical disease in which maintenance of this equilibrium is severely disturbed[20]. Since commensal bacteria are believed to be a crucial part of host homeostasis, recent studies have looked at effects of probiotics in recreating equilibrium[21]. Our group previously developed a probiotic combination designed to prevent infectious complications in critical illness based on anti-inflammatory and microbiota modulating capacities[22]. Five-day pretreatment with these multispecies probiotics attenuated bacterial translocation and

7 Deitch & Dayal, 2006
8 Clark & Coopersmith, 2007; Ammori, 2003
9 Katsube *et al.,* 2007; Basuroy *et al.,* 2006
10 Van Itallie & Anderson, 2006
11 Wu *et al.,* 2007
12 Yasuda *et al.,* 2006; Heller *et al.,* 2005; Abreu *et al.,* 2000
13 Coopersmith *et al.,* 2002; Yasuda *et al.,* 2007
14 Hotchkiss *et al.*, 2000
15 Tomson *et al.*, 2004
16 Kohler *et al.*, 2005
17 Clark & Coopersmith, 2007
18 Ding *et al.*, 2008
19 Solis *et al.*, 2002
20 Ammori, 2003
21 Guarner & Malagelada, 2003; Peran *et al.*, 2006; Alberda *et al.*, 2007
22 Timmerman *et al.*, 2007

PROBIOTICS, CONTRARY TO ANY EXPECTATIONS, DOUBLED THE MORTALITY COMPARED WITH PLACEBO

THE AIM WAS TO EXPLORE MECHANISMS BY WHICH PROBIOTICS AFFECT BARRIER FUNCTION

reduced the mortality in experimental AP in rats[23], but recently we also demonstrated in a double-blind clinical study that these probiotics, contrary to any expectations, doubled the mortality compared with placebo in 298 patients with predicted severe AP[24]. These results painfully showed the need to study mechanisms of action of probiotics in critical illnesses. The objective of this study was to characterize the intestinal mucosal barrier in experimental AP and to explore mechanisms by which multispecies probiotics affect barrier function under these circumstances. We found that probiotics maintained the mucosal barrier in AP by up-regulation of the rate-limiting step in glutathione (GSH) biosynthesis.

23 van Minnen *et al.*, 2007
24 Besselink *et al.*, 2008

MATERIALS AND METHODS

RATS

Male specific pathogen-free Sprague-Dawley rats (B&K, Sollentuna, Sweden, 250-350g, 50-70 days of age) were maintained under constant conditions with a 12-hour light/dark cycle and free access to water and standard rat pellets. Rats were acclimatized for one week prior to surgery and randomly allocated into five groups: 1) non-operated controls (n=5); 2) sham-procedure (n=12); 3) AP (n=12); 4) AP, placebo (n=12); 5) AP, probiotics (n=12). The experimental design (fig 1) was in accordance with guidelines of the Linköping University Animal Welfare Committee, following European legislation (2003/65/EC).

PROBIOTICS

The multispecies probiotics consisted of six viable, freeze-dried strains: *Lactobacillus acidophilus* (W70), *Lactobacillus casei* (W56), *Lactobacillus salivarius* (W24), *Lactococcus lactis* (W58), *Bifidobacterium bifidum* (W23), and *Bifidobacterium lactis* (W52) (previously classified as *Bifidobacterium infantis*) (*Ecologic® 641*, Winclove Bio Industries, Amsterdam, the Netherlands). Placebo, that consisted of the carrier of the probiotic product, i.e. cornstarch and maltodextran, was packed in identical coded sachets to guarantee blinding. Before daily administration, probiotic or placebo formulas were reconstituted in sterile water, for 15 min at 37°C. A single probiotic dose of 1.0 ml contained $5x10^9$ colony forming units (CFU) of bacteria.

SURGICAL PROCEDURES

Under general anesthesia (2% isoflurane), a permanent gastric cannula was fitted in all rats, as performed previously[25]. Animals were allowed to recover for four days prior to the start of daily probiotics or placebo administrations through the cannula and then after five days of pretreatment AP was induced as described by Schmidt *et al.* (1992). Briefly, pressure controlled (35<mmHg) retrograde infusion of 0.5 ml sterilized glycodeoxycholic acid (Sigma-Aldrich, Zwijndrecht, the Netherlands) into the biliopancreatic duct was followed by intravenous cerulein (5µg/kg/h, 1ml/h, for 6h, Sigma-Aldrich). During the sham procedure, cannulation of the biliopancreatic duct without infusion of glycodeoxycholic acid was followed by intravenous saline (1ml/h, 6h). Three animals needed to be excluded due to detachment of the gastric cannula and two due to anaesthesiological failures.

COLLECTION OF SAMPLES

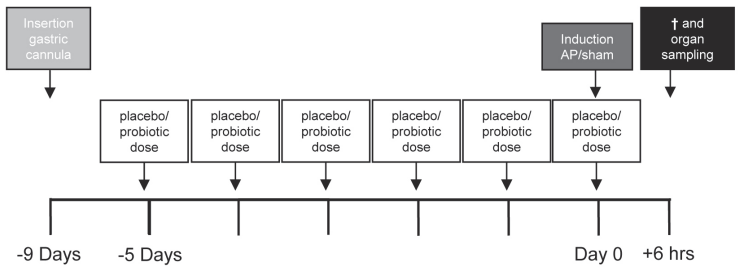
Whole blood was sampled by tail vein puncture, before start of treatment and before induction of acute pancreatitis. After cerulein or saline infusion, rats were anaesthetized and 15 cm of distal ileum, the pancreatic tail and whole blood were collected. Ten cm of ileum was used for Ussing chamber experiments and immediately

25 van Minnen *et al.*, 2007

FIGURE 1

Experimental design

At the start of the experiment, animals were fitted with a gastric cannula, except for control animals. Probiotics and placebo were administered daily to the probiotics and placebo group, starting 5 days prior to induction of acute pancreatitis (AP). At day 0, AP or sham-procedure was performed. After the six hours of cerulein infusion, animals were anesthetized for removal of organ samples.



submersed into ice-cold oxygenated Kreb's buffer (115mM NaCl, 1.25mM $CaCl_2$, 1.2mM $MgCl_2$, 2mM KH_2PO_4, and 25mM $NaHCO_3$, pH 7.35). The remainder was flushed with cold Kreb's buffer to remove adherent bacteria, stripped of the external muscle, freeze-dried and stored at -70°C until analyzed. Histological assessment verified that no bacteria remained associated with the tissue samples. Samples for histological and immunohistochemical examinations were formalin fixed, and embedded in optimum cutting temperature compound (Histolab, Västra Frölunda, Sweden). All analyses were run in duplicates.

USSING CHAMBER EXPERIMENTS

Mucosal permeability was measured as previously described[26]. Briefly, ileum, stripped of external muscle while immersed in Kreb's buffer, was mounted into Ussing chambers (Harvard Apparatus Inc., Holliston, MA, USA[27] where 9.6 mm^2 tissue was exposed to 3 ml (1.5 ml each half-chamber) circulating, oxygenated Kreb's solution at 37°C. The serosal buffer contained 10 mM glucose as energy source and was osmotically balanced by 10 mM mannitol in the mucosal buffer. Chambers contained agar-salt bridges to monitor potential difference across the tissue for vitality assessment. Baseline values for short circuit current (Isc), indicating net ion secretion, and conductance (passive ion flux), were recorded at equilibrium, 40 min after mounting.

26 Velin *et al.*, 2004
27 Grass & Sweetana, 1988

Transepithelial transport of macromolecules was assessed by measuring horseradish peroxidase (HRP) (Sigma-Aldrich), as model antigen, and ^{51}Cr-EDTA (Perkin-Elmer, Boston, MA, USA) flux, as paracellular probe. HRP and ^{51}Cr-EDTA were added to the mucosal side to a final concentration of 10^{-5} M and 34μC_i/ml, respectively. Serosal samples (300μl) were collected at 0, 30, 60, 90 and 120 min after start and were used to analyze transepithelial fluxes of ^{51}Cr-EDTA, expressed as cm/s·10^{-6}, using a gamma-counter (1282 Compugamma, LKB, Bromma, Sweden). HRP-activity was determined as previously described[28], and transepithelial HRP flux was expressed as pmol·cm^{-2}·h^{-1}. Permeability was calculated in 3 ileal samples per rat during the 30–120 min period for both markers.

To assess bacterial passage, fluorescent *E.coli* K12 (1x10^8 CFU/ml, Molecular Probes, Leiden, the Netherlands), killed by paraformaldehyde to stop reproduction without loss of antigenicity[29], were added after equilibration, to the mucosal side. After 120 min, the entire volume of serosal compartments was analyzed at 488 nm in a fluorimeter (Cary Eclipse, Varian, Victoria, Australia). One unit corresponds to 3.0·10^3 CFU/ml[30].

IMMUNOHISTOCHEMISTRY

Frozen ileum sections (5µm) of 4 rats per group were incubated with 5% bovine serum albumin, washed and incubated with a primary antibody (1:50 rabbit anti-rat occludin, mouse anti-rat claudin-1 or mouse anti-rat claudin-2; Zymed Laboratories, San Francisco, CA, USA) for 1 h at room temperature. Following extensive washes, slides were incubated with Alexa Fluor®488 goat anti-mouse or anti-rabbit immunoglobulin-G (1:500 dilution, Jackson ImmunoResearch Europe Ltd, London, United Kingdom) for 1 h at room temperature.

Apoptotic cells were detected by 'in-situ cell death detection kit' (Roche Diagnostics, Bromma, Sweden). Frozen ileum sections (5µm) were permeabilized in 0.1 mol/l sodium citrate for 2 min on ice and incubated in terminal-deoxynucleotidyl-transferase-mediated-dUTP-nick-end-labeling (TUNEL) reaction mixture for 1 h at 37°C.

All sections were counterstained with 0.5 µM 4′,6-diamidineo-2-phenylindole (DAPI) for 10 min, mounted in antifading Fluorescent Mounting Medium (DakoCytomation, Stockholm, Sweden) and examined using confocal imaging with a 2-photon BioRad Radiance 2000 microscope (Carl Zeiss, Jena, Germany), equipped with high numerical aperture (NA=1.4) 60× and 100× oil immersion objectives. Each test included negative controls. Image acquisition settings were identical for each experiment. Apoptotic rate was determined by counting the number of TUNEL$^+$ cells/100 epithelial cells in 4 sections from 4 rats per group.

28 Velin *et al.*, 2004
29 Wan *et al.*, 1993
30 Velin *et al.*, 2004

DNA-FRAGMENTATION ASSAY

Histone-associated DNA-fragmentation was determined in ileal homogenate corresponding to 50 μg freeze-dried mucosa as previously described[31], using Cell Death Detection ELISA ^{PLUS} (Roche Diagnostics). Results are normalized to protein content, as measured according to Bradford's method[32] and expressed as ratio to control animals.

HISTOLOGICAL MEASUREMENTS OF MUCOSAL DAMAGE

Coded ileal sections were haematoxylin-eosin (H&E) stained and the degree of mucosal damage was determined in 4 tissue sections per rat, by a pathologist blinded to the experimental design. Histopathological grading, from 0 (normal mucosa) to 5 (severe mucosal damage), was performed according to criteria by Chiu *et al.*(1970).

To confirm pancreatitis, histological analysis of H&E stained pancreatic sections was performed in 4 tissue sections per rat, utilizing Spormann's criteria[33].

LIPID PEROXIDATION

To assess oxidative damage, malondialdehyde (MDA) concentration was determined, using a lipid peroxidation assay (LPO-586; Byoxitech, OXIS International, Portland, OR, USA). Ileal mucosa was homogenized in 5 mM butylated hydroxytoluene to prevent sample oxidation. Supernatants were used to determine MDA levels according to manufacturer's instructions. Results were normalized to protein contents of the crude homogenates.

GLUTATHIONE ASSAY

To estimate the anti-oxidative capacity, reduced and oxidized GSH contents were determined in ileal tissue and plasma using a commercially available assay (Glutathione Assay Kit II, Merck Chemicals, Hull, United Kingdom). To ensure absence of adherent bacteria, samples were flushed with cold Kreb's buffer, and microscopically examined. Freeze-dried ileal mucosa was homogenized in acid medium (0.2 M 2-(N-morpholino)ethanesulphonic acid, 0.05 M phosphate, and 1 mM EDTA, pH 6.0), centrifuged (10 min, 10,000x*g*) and supernatants were collected. After protein determination, supernatants corresponding with 1 mg protein, and plasma aliquots were deproteinized with 5% metaphosphoric acid (Sigma-Aldrich Chemie BV) and 4 M triethanolamine (Sigma-Aldrich Chemie BV) and plasma samples were lyophilized.

Individual bacterial strains from the used probiotics were grown in de Man-Rogosa-Sharpe (MRS) broth at 37°C for 24 h, under strict anaerobic conditions. To determine GSH release during bacterial growth, samples were taken at 0, 6, and 24 h, centrifuged (4,000x*g* for 10 min at 4°C) and supernatants were collected. For determination of bacterial GSH content, bacteria were collected after 24 h of cultivation and disrupted by sonication (Bransonic 3200, Branson Ultrasonics b.v., Soest,

31 Trulsson *et al.*, 2002
32 Bradford, 1976
33 Spormann *et al.*, 1989

the Netherlands) on ice for 10 min with 3 sec cooling interval per min. Suspensions were centrifuged, yielding a cell-free extract.

Cell-free extracts, tissue supernatants and plasma samples were analyzed for total GSH according to the protocol provided by the manufacturer. To quantify oxidized GSH (GSSG), 2-vinylpyridine was added to the acidic medium to derivatize GSH. GSH levels were calculated by subtracting the amount of GSSG from the total GSH content and normalized to protein content.

CYSTEINE

Mucosal cysteine was determined using the spectrophotometric method developed by Gaitonde[34] and expressed as nmol/mg protein.

GLUTAMATE-CYSTEINE-LIGASE

Biosynthesis of GSH was analyzed by quantification of glutamate-cysteine-ligase (GCL, EC: 6.3.2.2) activity as previously described[35]. For determination of systemic GSH biosynthesis, erythrocytes were obtained by centrifugation of ETDA blood samples at 900*g* for 3 min and after washing 3 times with 5 volumes of cold isotonic NaCl solution. Erythrocytes were lysed by the addition of 50 mmol Tris-HCl buffer (pH 7.4), containing 1 mmol EDTA, and by sonication for 2 × 20 sec. The erythrocyte membranes were removed by centrifugation at 18,000*g* for 40 min. For determination of local intestinal mucosal GCL activity, ileal tissues were homogenized in 250 mM sucrose containing 20mM Tris, 1mM EDTA, 20 mM boric acid, 2 mM serine, pH 7.4. GCL activity was determined as the difference between γ-glutamylcysteine (GC) synthesis in unblocked and GC synthesis in samples blocked with 200 mM 5-sulfosalicylic acid dehydrate and expressed as nmol (GC)/min/mg protein.

mRNA EXPRESSION ANALYSIS

Total RNA was isolated from ileal mucosa using the RNeasy Midi Kit (Qiagen, Hilden, Germany) and spectrophotometrically quantified, showing A260/A280 ratios within normal range. Subsequently, the integrity of total RNA was checked by denaturing agarose gel electrophoresis. First strand cDNA was synthesized from total RNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) and quantitative RT-PCR was performed using the iCycler iQ system (BioRad). RT-PCR with mRNA-specific primers for the catalytic (GCLc) and modifier (GCLm) subunits of GCL and 18S rRNA as a reference gene was performed (GCLc-forward 5'-ggcgatgttcttgaaactctg-3', GCLc-reverse 5'-cagagggttgggtggttg-3'; GCLm-forward 5'-ctgactcacaatgacccaaaag-3', GCLm-reverse 5'-ttcaatgtcagggatgctttc-3'; 18S rRNA-forward 5'-aatcagttatggttcctttgtcg-3', 18S rRNA-reverse 5'-gctctagaattaccacagt-tatccaa-3'; Sigma-Aldrich, Zwijndrecht, The Netherlands) and mRNA levels were quantified using SYBR Green based detection.

34 Gaitonde, 1967
35 White *et al.*, 2003

Prior to real-time PCR analysis cDNA samples were diluted 1:25, except for 18S rRNA which was diluted 1:1000, with RNase-free water. PCR reactions were set up in a volume of 25 µl, containing 5 µl of diluted cDNA, 12.5 µl of 2x iQ SYBR Green Supermix (BioRad) and 300nM of the forward and reverse primer each. Thermal cycling conditions were 95°C for 3 min as initial denaturation and enzyme-activating step followed by 40 cycles of 95°C for 15sec denaturation, 60°C for 30sec annealing and 72°C for 30sec extension. After the amplification a melting curve analysis was performed by increasing the temperature by 0.5°C increments from 55°C to 95°C and measuring fluorescence at each temperature for a period of 10sec. All cDNA samples were analyzed in triplicate and each run contained a relative standard curve. Purified PCR products were used to generate the relative standard curves, consisting of serial dilutions. Levels of GCLc and GCLm mRNA were quantified using the comparative threshold cycle method, normalized to18S rRNA expression and expressed as ratio to controls (a.u.).

STATISTICAL ANALYSIS

Normal distribution was assessed using Shapiro–Wilk's test. Parametric values are presented as mean (SD). Statistical analysis was performed by ANOVA followed by Tukey's HSD test. Non-parametric values are given as median (25–75th inter-quartile range). Comparisons between two groups were done by Mann-Whitney U-test and between multiple groups by Kruskal-Wallis. Spearman's rank correlation coefficients were computed for correlation analyses. Considering Bonferroni's correction, $P<0.01$ was considered significant.

RESULTS

ACUTE PANCREATITIS INDUCED SEVERE ILEAL MUCOSAL BARRIER DYSFUNCTION

Mortality due to AP did not occur. Pancreatitis was confirmed by histological scoring[36] of pancreatic injury[37].

Pancreatitis induced increased permeability to HRP and ^{51}Cr-EDTA (fig 2A, B), accompanied by increase in baseline conductance, representing paracellular ion flux (fig 2C) and elevation of Isc, indicative of ion secretion (fig 2D). Moreover, transepithelial bacterial passage increased by as much as 7-fold in animals subjected to AP (fig 2E) and tissues from rats in the pancreatitis group responded to *E. coli* K12 added to the luminal buffer with an enhanced elevation in conductance (fig 2F). These data suggest a combined perturbation of paracellular and transcellular pathways.

To further characterize the paracellular pathway TJ protein expression was studied in tissue sections. In sham-operated animals, occludin was localized in the cytoplasm of epithelial cells and along the basolateral membrane with an enrichment of occludin at the apical surface (fig 3). In AP partial disruption of occludin was seen in crypts as well as in villi. Claudin-1 staining pattern was diffuse with predominantly intracellular localization in sham-operated animals (fig 3). AP caused decreased staining intensity and aggregation of claudin-1 within the cytosol. Claudin-1 was not detected near areas of epithelial disruption, suggesting that detachment of enterocytes was preceded by loss of claudin-1. Contrary to occludin and claudin-1, the pore-forming TJ protein, claudin-2[38], was only scarcely detectable in crypts of sham-operated animals, whereas rats from the AP group showed intense staining of claudin-2 both in crypt and surface epithelium (fig 3).

It is reported that intestinal epithelial apoptosis contributes to mucosal barrier dysfunction[39]. Confocal microscopy with immunofluorescent TUNEL staining (fig 4A) revealed AP-induced epithelial cell apoptosis compared to sham animals[40]. Mucosal DNA-fragmentation corroborated these findings (fig 4B). Moreover, DNA-fragmentation strongly correlated with bacterial passage (fig 4C), ^{51}Cr-EDTA flux (r=0.93) and tissue conductance (r=0.87), which supports the hypothesis that epithelial cell apoptosis disrupts barrier integrity.

Six hours after induction of pancreatitis, intestinal injury (fig 5A) was characterized by villus denudation, lamina propria disintegration and ulceration (fig 5B), resembling intestinal ischemia-reperfusion injury[41], which is associated with ROS release, epithelial apoptosis and TJ disruption[42]. Therefore, oxidative stress-induced lipid peroxidation was quantified, which was indeed found to be elevated after induction of AP (fig 5C) and also showed a strong positive correlation with barrier dysfunction[43].

36 Spormann *et al.*, 1989
37 sham-operated 0 (0-0) *vs.* after induction of pancreatitis 3 (2-5.1); $P<0.001$
38 Van Itallie & Anderson, 2006
39 Yasuda *et al.*, 2006; Heller *et al.*, 2005; Abreu *et al.*, 2000
40 25.8 (24.4-26.3) *vs.* 2.60 (2.47-2.73) TUNEL$^+$cells/100 epithelial cells; $P<0.001$
41 Chiu *et al.*, 1970
42 Wu *et al.*, 2007; Katsube *et al.*, 2007; Basuroy *et al.*, 2006
43 ^{51}Cr-EDTA flux r=0.83, bacterial passage r=0.88

PROBIOTICS PREVENTED ACUTE PANCREATITIS-INDUCED BARRIER DYSFUNCTION

No rats receiving probiotics showed signs of diarrhea or loss of appetite during the pretreatment period. Increase in animal weight was similar in all groups[44]. Five days of pretreatment with probiotics abolished the deleterious effects of AP on numerous parameters of barrier function. AP-induced increase in ileal permeability to HRP (fig 2A) and ^{51}Cr-EDTA (fig 2B) as well as tissue conductance (fig 2C) was normalized after probiotic pretreatment. Elevation in tissue conductance after adding *E.coli* K12 was 40% smaller in tissues from probiotic treated rats compared to placebo (fig 2F). In contrast, there were no inhibitory effects of probiotics on AP-induced elevation of Isc (fig 2D).

Probiotics also modified the localization of TJ proteins. AP-associated partial disruption of the distribution of occludin in crypts and villi was prevented and re-distribution to the apical surface was apparent in crypts of probiotic treated animals (fig 3). In both claudin-1 and -2 staining patterns the AP-induced deleterious effects were reduced by probiotic pretreatment (fig 3).

Furthermore, probiotics attenuated AP-induced epithelial cell apoptosis, showing a 70% reduction in apoptotic rate[45] (fig 4A), which was also demonstrated by analysis of mucosal DNA-fragmentation (fig 4B). Histological scoring demonstrated that probiotics ameliorated pancreatitis-induced mucosal damage[46] (fig 5A) and normalized mucosal lipid peroxidation (fig 5C).

BENEFICIAL EFFECT OF PROBIOTICS BY INCREASING MUCOSAL GLUTATHIONE

The decline in mucosal lipid peroxidation after probiotic pretreatment may have resulted from either reduced amounts of ROS, or enhanced anti-oxidative capacity. Therefore, we quantified mucosal oxidized glutathione (GSSG) and GSH in thoroughly rinsed ileal mucosal tissues. Probiotics attenuated AP-induced elevation in GSSG (fig 6A), prevented depletion of mucosal GSH (fig 6B) and normalized the GSH/GSSG ratio (fig 6C). Of note, mucosal GSH/GSSG ratios showed an inverse correlation with DNA-fragmentation (fig 6D) and mucosal barrier dysfunction (fig 6E, F). Most interestingly, pretreatment with probiotics induced increased levels of GSH, also in comparison with healthy control animals (fig 6B).

PRODUCTION OF GLUTATHIONE BY THE INDIVIDUAL PROBIOTIC STRAINS

Since mucosal GSH is partially dependent on uptake of dietary GSH[47], we quantified intrabacterial GSH of the separate probiotic strains, as well as GSH levels in medium after 6 and 24 hours of strictly anaerobic cultivation. Only *B. bifidum, B. lactis* and *L. acidophilus* contained abundant intracellular GSH (table 1). GSH in cultivation medium increased over time, except for *Lc. lactis.*

44 sham-operated 22.3 (1.09) *vs.* AP 21.9 (1.11) *vs.* placebo 20.6 (0.82) *vs.* probiotics 20.5 (0.61)
45 8.85 (8.60-9.15) *vs.* placebo 31.75 (31.60-32.90) TUNEL$^+$ cells/100 epithelial cells; $P<0.001$
46 1.0 (1.0-1.25) *vs.* placebo 4.0 (4.0-4.0); $P<0.001$
47 Hagen *et al.*, 1990

LOCAL MUCOSAL BIOSYNTHESIS OF GLUTATHIONE

Mucosal GSH is, besides dietary uptake, also dependent on biosynthesis, which is regulated by availability of cysteine and GCL activity[48]. Mucosal cysteine levels did not differ significantly between the groups (fig 7A). GCL activity, however, was affected by AP and by probiotics pretreatment. AP *per se* increased GCL activity compared to sham, but the most abundant increase was seen in the probiotics group which showed a 10-fold increase compared to controls (fig 7B).

Because GCL is composed of both a catalytic and modulatory subunit[49], the increase in GCL activity in this study could be due to enhanced expression of either GCLm and/or GCLc. Therefore, quantitative real-time PCR was performed to monitor changes in GCLc and GCLm message abundance. The level of mRNA expression of the reference gene, 18S, was comparable between all groups. When normalized to 18S, GCLc levels in probiotic pretreated rats were 6.78 (0.95) a.u., which was 1.8 fold higher than in placebo treated rats (3.83 (0.49) a.u., fig 7C, D). Similarly, levels of GCLm mRNA after probiotic pretreatment (10.3 (2.56) a.u) were 2.7 fold higher than average values seen in placebo treated rats (3.88 (0.75) a.u). The increase in GCLm mRNA expression was less pronounced in the AP and placebo groups when compared to sham operated animals[50] (fig 7C, E). These data suggest that the enhanced GCL activity after probiotic pretreatment may be due to increased gene expression in the ileal mucosa.

PROBIOTICS INCREASE SYSTEMIC GLUTATHIONE LEVELS

To gain insight into the anti-oxidative capacity prior to induction of AP, plasma GSH levels before treatment, before induction of AP and at time of termination were determined. In the course of the 5 days of the pretreatment period, plasma GSH levels showed a 2 fold increase in probiotic pretreated animals (fig 8A). This was in contrast to rats receiving placebo, in which plasma GSH levels did not differ significantly after 5 days of pretreatment. This is in keeping with the increase in GCL activity in red blood cells (RBC) after 5 days of pretreatment with probiotics (fig 8B). Correlation analyses between pancreatitis-induced oxidative damage, as measured by ileal lipid peroxidation and GCL activity immediately before induction of pancreatitis, suggested that GCL activity greater than 5 nmol/min/mg protein was protective against oxidative injury (fig 8C). Furthermore, GCL activity in RBCs immediately before induction of acute pancreatitis correlated inversely with parameters of mucosal barrier dysfunction in animals subjected to AP[51] (fig 8D, E). Not surprisingly, GCL activity also correlated positively with ileal GSH levels in animals subjected to AP (r=0.82, fig 8F).

48 Meister & Anderson, 1983
49 Lu, 2008
50 3.07 *vs.* AP 4.68 a.u.; 3.07 *vs.* placebo 3.88 a.u., respectively
51 bacterial passage: r=-0.80, ^{51}Cr-EDTA: r=-0.86

TABLE 1

Intracellular GSH contents of the probiotic strains and GSH levels in medium at different time points after start of cultivation

	Intracellular GSH (nmol/mg protein)	GSH content in culture medium (nmol/ml supernatant)		
Bacterial strain		**0 hour**	**6 hours**	**24 hours**
B. bifidum	0.37 (0.002)	0.00 (0.000)	0.10 (0.006)	0.51 (0.006)
L. salivarius	0.11 (0.002)	0.00 (0.000)	0.08 (0.004)	0.15 (0.006)
B. lactis	0.01 (0.002)	0.00 (0.000)	0.00 (0.000)	0.01 (0.008)
L. casei	0.09 (0.004)	0.00 (0.000)	0.00 (0.000)	0.01 (0.002)
Lc. lactis	0.04 (0.014)	0.00 (0.000)	0.00 (0.000)	0.00 (0.000)
L. acidophilus	0.14 (0.005)	0.00 (0.000)	0.04 (0.002)	0.19 (0.002)

Bifidobacterium (B.); Lactobacillus (L.); Lactococcus (Lc.); glutathione (GSH)

Mean (SD), n=4 separate experiments

FIGURE 2

Probiotics prevented acute pancreatitis-induced ileal permeability but not ion secretion

After 5 days of pretreatment with placebo (pla, n=12) or probiotics (pro, n=12), rats were subjected to acute pancreatitis (AP, n=12), a sham-procedure (n=12) or not operated (control, n=5). Ileal segments were mounted in Ussing chambers and (A) horseradish peroxidase (HRP) and (B) ^{51}Cr-EDTA flux were studied for two hours. (C) Baseline conductance, (D) baseline short circuit current (Isc), (E) passage of *Escherichia coli* K12 and (F) elevation of conductance during one hour after challenge with *E. coli* were measured. The graphs show average (±SD). The data were collected from independently acquired sets of 3 tissue segments per rat. Comparisons were performed using ANOVA followed by Tukey's HSD.

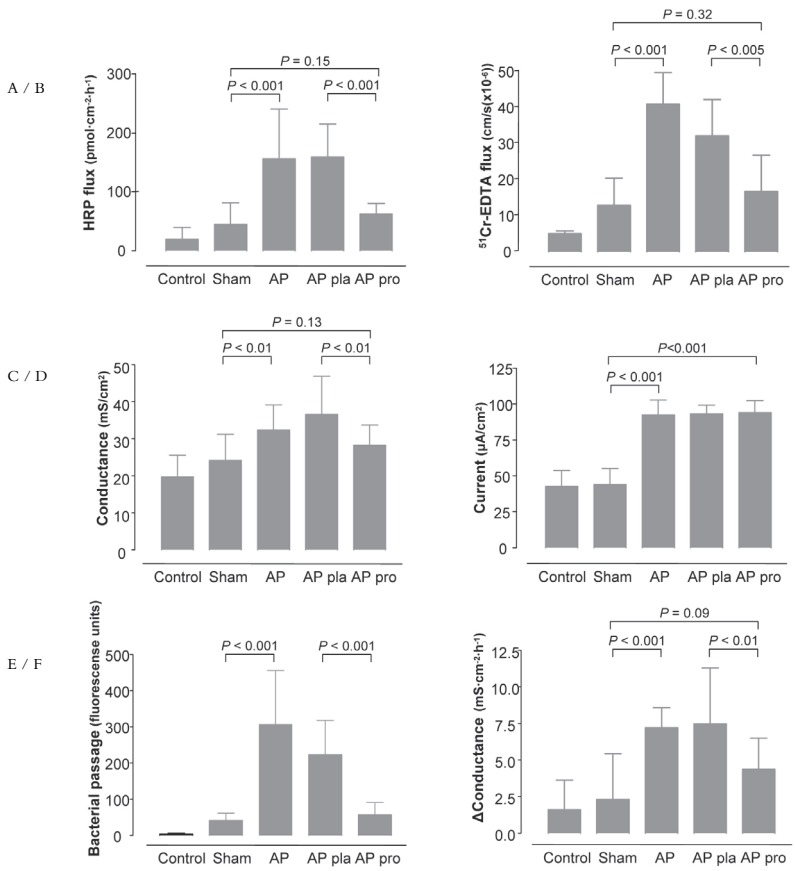


FIGURE 3

Probiotics prevented disruption of tight junction proteins

After 5 days of pretreatment with placebo (pla) or probiotics (pro), rats were subjected to acute pancreatitis (AP), or a sham-procedure. Ileal sections were stained with occludin, claudin-1 or -2 antibodies (green), counterstained with DAPI (blue) and visualized by confocal laser scanning microscopy. Bar = 500 μm. The higher magnification (100x/1.30) images shown in the insets are typical details of crypts (left) and villi (right). Probiotics prevented the deleterious effects of AP on occludin and caused redistribution of occludin to the apical surface (arrowhead). Acute pancreatitis-induced detachment of epithelial cells seems to be preceded by loss of claudin-1 (arrowhead) and was reduced by probiotic pretreatment; though probiotics could not prevent the AP-induced formation of aggregates of claudin-1 in the cytosol (arrowhead). Probiotics prevented AP-associated up-regulation of claudin-2 in both crypts and villi (arrowhead). The patterns of staining are typical of that seen in 4 sections of 4 rats per group.

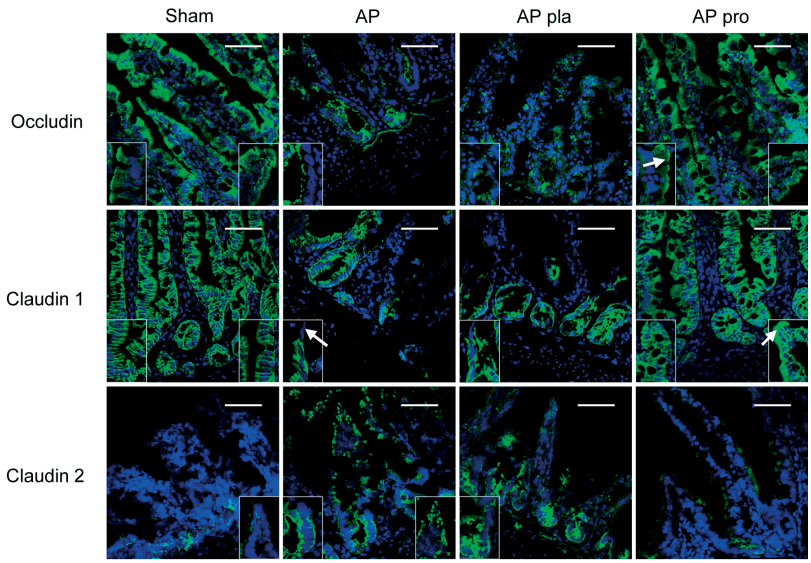


FIGURE 4

Probiotics reduced pancreatitis-associated intestinal apoptosis

After 5 days of pretreatment with placebo or probiotics, rats were subjected to acute pancreatitis, or a sham-procedure. (A) Sections of ileum were TUNEL stained. The results shown are typical images from 4 sections of 4 rats per group. Bar = 200 μm. (B) DNA-fragmentation (control n=5, sham n=12, AP n=12, AP pla n=12 and AP pro n=12). The graph shows the average (±SD). Comparisons were performed using ANOVA followed by Tukey's HSD. (C) Positive correlation between intestinal apoptosis and *Escherichia coli* K12 passage was computed using Spearman's rank correlation coefficients.

A

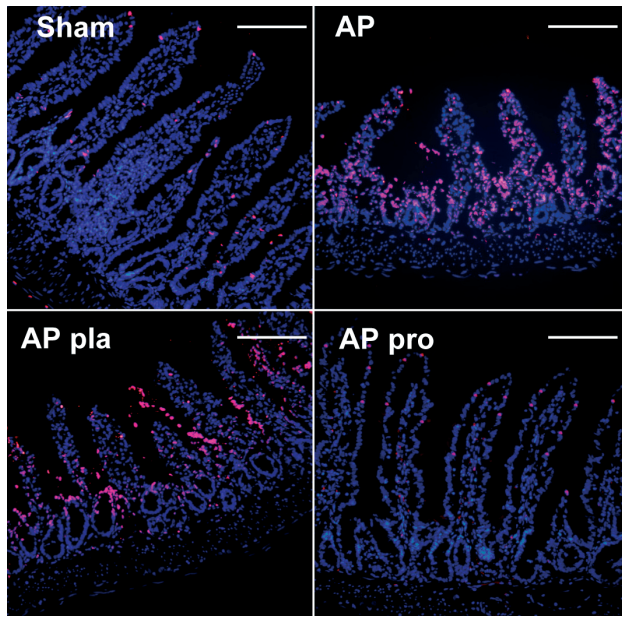
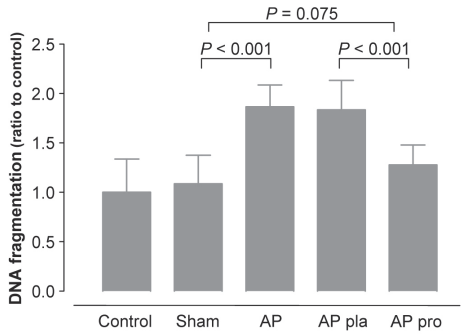


FIGURE 4

B


P = 0.075
P < 0.001
P < 0.001
DNA fragmentation (ratio to control)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Sham
AP
AP pla
AP pro

C

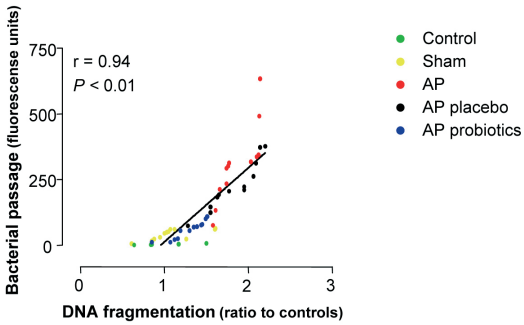
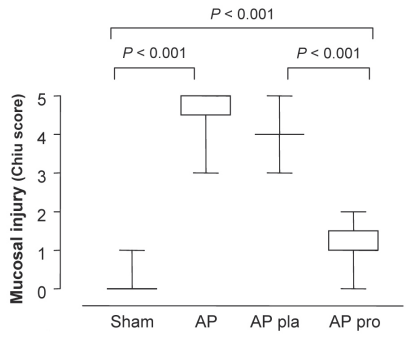

Bacterial passage (fluorescense units)
750
500
250
0
r = 0.94
P < 0.01
0
1
2
3
DNA fragmentation (ratio to controls)
Control
Sham
AP
AP placebo
AP probiotics

FIGURE 5

Probiotics attenuated acute pancreatitis-associated mucosal damage

After 5 days of pretreatment with placebo (pla, n=12) or probiotics (pro, n=12), rats were subjected to acute pancreatitis (AP, n=12), or a sham-procedure (n=12). (A) Sections of ileum were H&E stained and graded according to Chiu *et al.* (1970). The graph shows median (±range). Comparisons were performed using Kruskal-Wallis followed by Mann–Whitney *U* test. (B) Compared with sham-operated animals (Sham), acute pancreatitis (AP) caused widespread destruction of villi. Placebo treated animals (AP pla) also showed a severe degree of mucosal damage. Probiotic animals (AP pro) showed extensive epithelial lifting, but with intact epithelium. The mucosal damage is typical of that seen in 4 sections from 12 rats per group. Bar = 200 μm. (C) Lipid peroxidation (MDA levels) (control n=5, sham n=12, AP n=12, AP pla n=12 and AP pro n=12). The graph shows the average (±SD). Comparisons were performed using ANOVA followed by Tukey's HSD.

A



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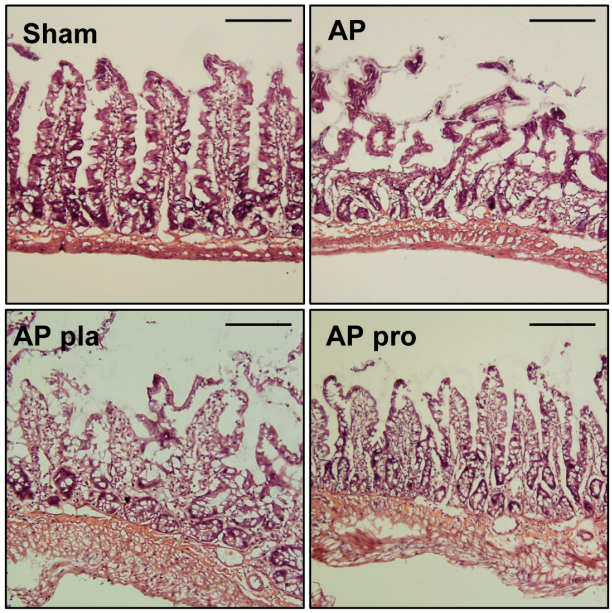
Sham
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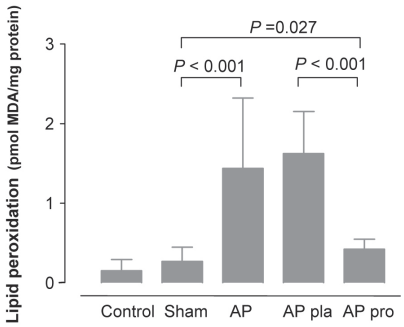

Lipid peroxidation (pmol MDA/mg protein)
3
2
1
0
P =0.027
P < 0.001
P < 0.001
Control
Sham
AP
AP pla
AP pro

FIGURE 6

Probiotics enhanced mucosal glutathione levels

After 5 days of pretreatment with placebo (pla, n=12) or probiotics (pro, n=12), rats were subjected to acute pancreatitis (AP, n=12), a sham-procedure (n=12) or not operated (control, n=5). Six hours after induction of the AP or sham-procedure, mucosal (A) oxidized glutathione levels (GSSG), (B) reduced glutathione levels (GSH) and (C) GSH/GSSG ratios were determined. The graphs show average (±SD). Comparisons were performed using ANOVA followed by Tukey's HSD. Correlation analyses revealed an inverse correlation between GSH/GSSG ratio and (D) DNA-fragmentation, (E) ileal permeability to *Escherichia coli* K12 and (F) ^{51}Cr-EDTA flux. Spearman's rank correlation coefficients were computed for correlation analyses.

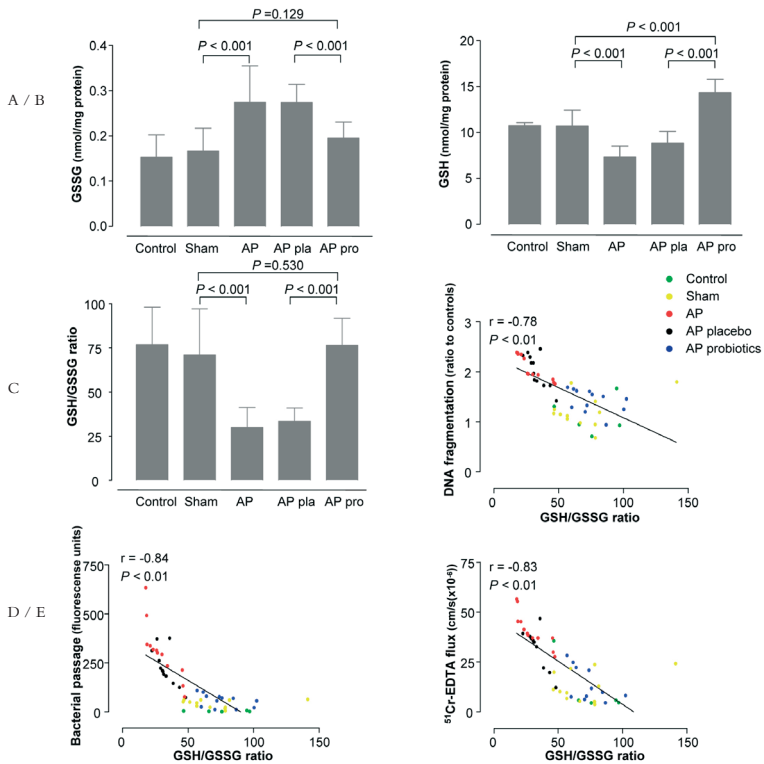
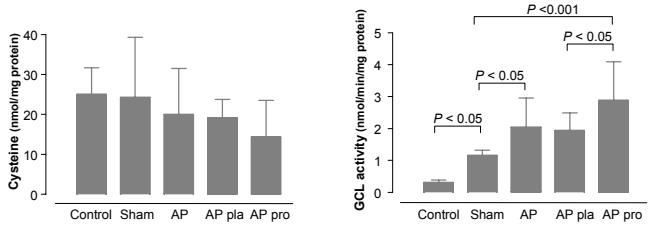


FIGURE 7

Probiotics have no effect on cysteine availability, but induce glutamate-cysteine-ligase activity

After 5 days of pretreatment with placebo (pla, n=12) or probiotics (pro, n=12), rats were subjected to acute pancreatitis (AP, n=12), a sham-procedure (n=12) or not operated (control, n=5). Six hours after induction of the AP or sham-procedure, tissue cysteine availability (A) and mucosal glutamate-cysteine-ligase (GCL) activity (B) were determined in ileum samples. (C) RT-PCR was conducted on ileal mRNA. PCR products of specific primers for the catalytic (GCLc, 65bp) and the modulatory (GCLm, 81bp) subunit of GCL and for 18S rRNA (65bp) as control were identified on 2.5% agarose gel, using a GeneRuler 50 bp DNA Ladder (Fermentas GMBH, St. Leon-Rot, Germany). mRNA expression of (D) GCLc and (E) GCLm. Data are normalized to 18S rRNA expression and expressed as ratio to control animals. The graphs show average (±SD). All analyses were run in triplicates. Comparisons were performed using ANOVA followed by Tukey's HSD.

A / B



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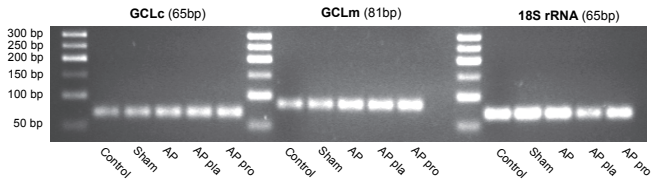


D / E

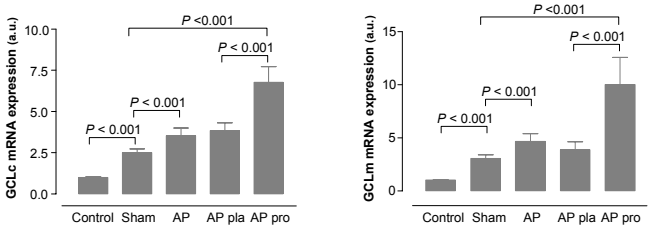
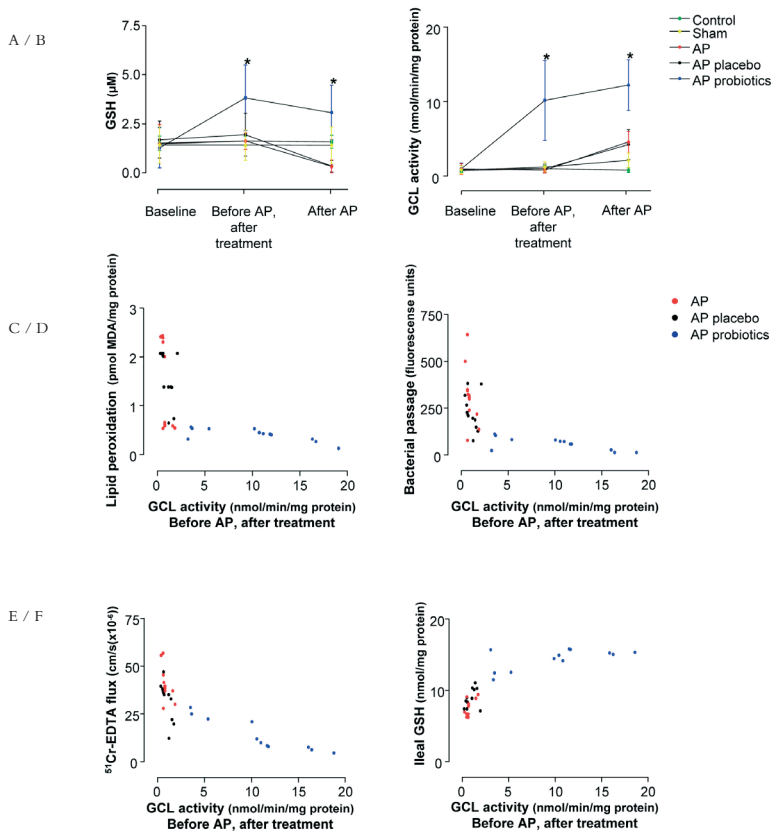


FIGURE 8

Probiotics induce systemic increase in GSH levels and GCL activity

After 5 days of pretreatment with placebo (pla, n=12) or probiotics (pro, n=12), rats were subjected to acute pancreatitis (AP, n=12), a sham-procedure (n=12) or not operated (control, n=5). Whole blood was sampled 1) before treatment, 2) after 5 days of pretreatment, immediately before induction of AP and 3) six hours after induction of AP or sham-procedure. Time course of plasma GSH levels (A) and GCL activity in red blood cells (B) was monitored. The graphs show average (±SD). All analyses were run in duplicates. Comparisons were performed using ANOVA followed by Tukey's HSD. *P<0.001, probiotics *vs.* placebo. Associations between (C) ileal lipid peroxidation, (D) bacterial passage, (E) ^{51}Cr-EDTA flux, (F) ileal GSH content six hours after induction of AP and GCL activity in red blood cells immediately before subjection to AP.



PRETREATMENT WITH PROBIOTICS STIMULATES MUCOSAL GLUTATHIONE BIOSYNTHESIS AND CONSEQUENTLY NORMALIZES ACUTE PANCREATITIS-INDUCED BARRIER DYSFUNCTION

THE APPROPRIATE CLINICAL USE OF PROBIOTICS WOULD BE A PREVENTIVE APPROACH TO IMPROVE DEFENSE AGAINST AN EXPECTED OXIDATIVE ATTACK

DISCUSSION

The present study is the first to demonstrate that pretreatment with multispecies probiotics increases mucosal GSH levels and stimulates GSH biosynthesis in the ileum, resulting in attenuated oxidative mucosal damage. Furthermore, normalization of GSH/GSSG ratios strongly correlated with improved barrier function. Therefore, increased mucosal GSH levels represent a candidate mechanism underlying the protection against barrier dysfunction afforded by pretreatment with probiotics in experimental AP.

GSH synthesis was upregulated in probiotic pretreated rats, as demonstrated by enhanced GCL activity and increased mRNA expression of both of the GCL subunits, shown herein. GSH plays a pivotal role in maintenance of the redox balance (expressed as GSH/GSSG ratios), preventing oxidative damage[52] and maintaining mucosal barrier, which was reflected by the inverse correlation between mucosal GSH/GSSG ratios and parameters of barrier dysfunction. Two factors directly associate with mucosal GSH: dietary GSH levels[53] and GSH biosynthesis, of which the latter is in turn dependent on cysteine availability and GCL activity[54]. First, it has been reported that certain probiotics contain and release GSH[55] and Peran *et al.* (2006) previously showed increased intestinal GSH following oral administration of *Lactobacillus fermentum* in experimental colitis. Our present *in vitro* experiments showed strain specific differences in intracellular GSH content within the range previously reported[56]. Moreover, time-dependent GSH release was found during anaerobic cultivation, which was abundant in *B. bifidum*, *B. lactis* and *L. acidophilus.* Nevertheless, considering an estimated GSH production of 31.0 nmol GSH by the total administered probiotic dose[57] compared with an estimated total increase in small intestinal GSH of 1190 nmol[58], bacterial GSH could only partially account for the rise in ileal GSH content. Consequently, the possibility of local GSH biosynthesis was investigated. The present study did not show significant differences in mucosal cysteine, implying that cysteine availability was not an important discriminating factor. On the other hand, we found enhanced GCL activity and expression of the GCL subunits GCLc and GCLm in probiotic-treated animals leading to a significant increase in GSH contents. Although the contribution from the probiotic bacteria may be higher than calculated because of colonization and expansion, it is conceivable that enhanced CGL activity in the intestinal mucosa was the major factor contributing to the increased ileal GSH content.

Interestingly, correlation analysis between GCL activity and parameters of mucosal barrier failure in animals subjected to AP, suggested the existence of a threshold GCL activity above which mucosal protection against oxidative stress is functional. This may explain that the relatively small increase in GCL activity between the

52 Deitch *et al.*, 1994
53 Hagen *et al.*, 1990
54 Meister & Anderson, 1983
55 Musenga *et al.*, 2007; Peran *et al.*, 2007
56 Musenga *et al.*, 2007
57 17.9 nmol intrabacterial GSH (mean 0.13 nmol GSH/mg protein) + 13.1 (1.53) nmol GSH secreted in 5 days; calculated from Table 1
58 small intestinal length 90 cm, mucosal protein content 2.4 mg/cm (n=6); pretreatment yielded increase in ileal GSH of 5.5 nmol/mg protein; placebo 8.8 *vs.* probiotics 14.3 nmol/mg protein, fig 6B

probiotic and the placebo pretreated groups resulted in considerable protection, whereas the rise in GCL activity between the sham and the AP group did not ameliorate the AP-induced damage. This hypothesis is supported by the correlation between GCL activity and ileal GSH content; the latter was only above a certain threshold of GCL activity able to withstand the deleterious effects of AP.

As previous experimental studies have shown that GCL gene expression is upregulated both after low dose H_2O_2[59] and after administration of weak inducers of oxidative stress[60], the increase in GCL activity found in the present study could be indicative of cellular stress as a mechanistic factor. Administration of probiotics may have caused a minor oxidative assault, e.g. intracellular accumulation of short-chain fatty acids produced by the bacteria, thereby inducing increased capacity of antioxidant enzymes, preconditioning the mucosa for a major oxidative attack during AP. This hypothesis is further supported by the found increase in systemic GCL activity in the probiotic pretreated group, which was markedly enhanced already before the induction of AP. At first glance, it may seem contradictory to the current study that the recent placebo-controlled trial by Besselink *et al.* (2008), demonstrated increased incidence of bowel ischemia after administration of probiotics in the acute phase of severe AP. However, keeping in mind that enteral probiotics caused low dose oxidative stress, probiotics administered after the onset of AP may act as an extra oxidative burden in an already critically affected redox system[61] thereby, causing increased oxidative stress-induced damage and ischemia.

During critical illness oxidative stress disrupts TJs[62], which are crucial in determining epithelial barrier properties[63], as illustrated here by the AP-induced breach in barrier function. Disruption of TJs results in increased permeability to luminal antigens and bacteria that promote release of pro-inflammatory cytokines which further deteriorates mucosal barrier function[64]. This is in keeping with our results that are the first to show AP-induced disruption of the claudin-1 distribution together with up-regulation of claudin-2, which is also the case in inflammatory bowel disease and destabilizes TJs[65]. Immunostaining revealed that pretreatment with probiotics maintained TJ integrity with a normal distribution of claudin-1 and -2. The finding of Yasuda and colleagues (2006), that AP did not have deleterious effects on occludin, is in contrast with our results. This may, however, be explained by differences in the model of AP used.

Apoptosis is the major mode of cell death during intestinal ischemia/reperfusion[66] and exerts deleterious effects on mucosal barrier function and survival[67]. Yan *et al.* (2007) previously showed that soluble proteins produced by *Lactobacillus* strains protect epithelial cells from cytokine-induced apoptosis. Here, we found that probiotics, which induced GSH biosynthesis, normalized AP-induced epithelial cell apoptosis. In addition, we were able to demonstrate a positive correlation between mucosal DNA-fragmentation and barrier dysfunction, providing further evidence

59 Ding *et al.*, 2008
60 Solis *et al.*, 2002
61 Ammori, 2003
62 Katsube *et al.*, 2007; Basuroy *et al.*, 2006
63 Van Itallie & Anderson, 2006
64 Clark & Coopersmith, 2007
65 Van Itallie & Anderson, 2006; Zeissig *et al.*, 2007
66 Wu *et al.*, 2007
67 Yasuda *et al.*, 2006; Heller *et al.*, 2005

that oxidative stress plays an important role in induction of epithelial apoptosis and subsequent loss of barrier function.

Contrary to the effects on permeability, pretreatment with probiotics showed no effect on ion secretion in our experiment. These findings emphasize the divergent regulation of cellular secretory and barrier functions. It has been reported that epithelia respond rapidly to pathogenic bacteria with altered ion secretion[68], indicating that "flushing out" may be a defense mechanism against the threat of invasion of the mucosa. In this regard, it is perhaps advantageous that probiotics do not inhibit these beneficial adaptive responses to an infectious threat.

In conclusion, the present study is to our knowledge the first to show that pretreatment with multispecies probiotics, stimulates mucosal GSH biosynthesis and consequently normalizes AP-induced barrier dysfunction and attenuates epithelial cell apoptosis and disruption of TJs in a model of AP. In addition, our data demonstrate strong inverse correlations between mucosal GSH/GSSG ratios and mucosal barrier dysfunction. This further supports the functional relevance of this endogenous antioxidant and gives novel insights into the mechanisms of probiotics. However, as the used compound is a multispecies combination of probiotic strains, it is worth noting that the found effects depend on the combination of the applied bacteria. Additional studies will be necessary to elucidate the effects of each separate strain as well as possible synergistic effects of this specific combination of probiotics.

In addition, the role of oxidative stress has been evaluated in experimental models of acute pancreatitis[69] and it should be emphasized that oxidative stress and excessive ROS generation are early features in AP and consequently a difficult target for clinical prophylaxis to prevent a severe course of the disease. This has recently been shown in a randomized controlled trial, utilizing intravenous antioxidant (n-acetylcysteine, selenium, vitamin C) therapy, where the results in AP patients were not that encouraging[70]. However, oxidative stress is not only involved in the early stage of AP, but also in the course of the disease and may for that reason be a target for therapy at later stages of the disease. Nevertheless, as the probiotics used in the current study showed severe adverse effects in intensive care AP patients[71], and since the present effects on GCL activity most likely resulted from a mild oxidative stress, this combination of probiotics is not a defendable treatment option in critically ill patients. Therefore, the appropriate clinical use of multispecies probiotics would be a preventive approach to improve defense against an expected oxidative attack, such as before elective major abdominal surgery[72] or maintenance treatment in IBD and pouchitis[73].

ACKNOWLEDGEMENTS

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68 Resta-Lenert & Barrett, 2002
69 Rau *et al.*, 2000
70 Siriwardena *et al.*, 2007
71 Besselink *et al.*, 2008
72 Sugawara *et al.*, 2006
73 Kruis *et al.*, 2004; Gionchetti *et al.*, 2000

6

Probiotics enhance pancreatic glutathione biosynthesis and reduce oxidative stress in experimental acute pancreatitis.

Probiotics reduce oxidative stress in acute pancreatitis

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L Paul van Minnen [2] Ger T Rijkers [2]
Harro M Timmerman [2] Lennart E Franzén [3]
Hein G Gooszen [2] Louis M A Akkermans [2]
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ABSTRACT

OBJECTIVE

Factors determining severity of acute pancreatitis (AP) are poorly understood. Oxidative stress causes acinar cell injury and contributes to the severity, while prophylactic probiotics ameliorate experimental pancreatitis. Our objective was to study how probiotics affect oxidative stress, inflammation and acinar cell injury during the early phase of AP.

DESIGN

53 Male Spraque-Dawley rats were randomly allocated into groups: 1) control, 2) sham procedure, 3) AP, no treatment, 4) AP, probiotics and 5) AP, placebo. AP was induced under general anesthesia by intraductal glycodeoxycholate infusion (15 mM) and intravenous cerulein (5μg/kg/hr, for 6 hours). Daily probiotics or placebo were administered intragastrically, starting 5 days prior to AP. After cerulein infusion, pancreas samples were collected for analysis including lipid peroxidation, glutathione, glutamate-cysteine-ligase activity, histological grading of pancreatic injury and NF-κB activation.

RESULTS

The severity of pancreatic injury correlated to oxidative damage (r=0.9) and was ameliorated by probiotics[1]. AP-induced NF-κB activation was

1 probiotics 1.5 *vs.* placebo 5.5; *P*=0.014

reduced by probiotics[2]. Probiotics attenuated AP-induced lipid peroxidation[3]. Not only was AP-induced glutathione depletion prevented[4], probiotic pretreatment even increased glutathione compared with sham rats[5]. Biosynthesis of glutathione (glutamate-cysteine-ligase activity) was enhanced in probiotic pretreated animals.

CONCLUSIONS

Probiotics enhanced the biosynthesis of glutathione, which may have reduced activation of inflammation and acinar cell injury and ameliorated experimental AP, via a reduction in oxidative stress.

2 probiotics 0.20 *vs.* placebo 0.53 OD_{450nm}/mg nuclear protein; $P<0.001$
3 probiotics 0.25 *vs.* placebo 0.51 pmol MDA/mg protein; $P<0.001$
4 probiotics 8.81 *vs.* placebo 4.1 µmol/mg protein, $P<0.001$
5 probiotics 8.81 *vs.* sham 6.18 µmol/mg protein, $P<0.001$

INTRODUCTION

Acute pancreatitis (AP) is a common condition of which clinical manifestations may range from a mild, self limiting disease to an inflammatory process with life threatening complications[6]. The severity of AP is difficult to predict at hospitalization, since underlying factors determining severity remain unclear. While evidence suggests that oxidative stress may be an important determinant of disease severity, little yet is known about the precise mechanisms and the extent of involvement[7].

Oxidative stress can be defined as an imbalance between cellular production of oxidants (reactive oxygen species, ROS) and the anti-oxidative capacity and is known to cause acinar injury in the early course of AP[8]. Normally, ROS are generated by aerobic cells and are readily removed by endogenous free radical scavenging mechanisms, but anti-oxidant defense systems of the cells can be overwhelmed by large quantities of ROS, with oxidative stress as a result[9].

The initial stage of AP is characterized by interstitial oedema - reducing tissue perfusion and oxygenation - and infiltration of neutrophils, which release large amounts of reactive oxygen species (ROS) into the pancreas[10]. In a rat model of AP Rau *et al.* (2000) demonstrated oxidative damage, in the form of lipid peroxidation, and acinar injury within 5 minutes after induction of AP. Furthermore, oxidative stress activates NF-κB[11], generating an inflammatory response, which attracts more oxidative stress-generating neutrophils, causing a vicious circle in AP[12].

Glutathione (GSH) plays an important role as part of an intracellular defense system to counteract potentially negative effects of ROS and its concentration mainly depends on *de novo* synthesis by glutamate-cysteine-ligase (GCL)[13]. In addition, GSH depletion has been found in early experimental AP to correlate with the extent of pancreatic injury[14].

The approach to modify the intestinal microbiota by oral intake of live bacteria to prevent intestinal disorders has been of growing interest[15]. It has been suggested that probiotics have antibacterial and immunomodulatory effects[16]. In addition, probiotics have been shown to increase GSH levels [17] and to reduce intestinal oxidative stress in several experimental models[18]. Furthermore, there is evidence showing that probiotics ameliorate morphological severity[19] and pancreatic DNA damage[20] in experimental AP. On the contrary, opposed to any expectations, our group recently found that probiotics, administered as treatment, more than doubled the relative risk of mortality in severe AP patients, which was probably related to an increased

6 Frossard *et al.*, 2008
7 Pandol *et al.*, 2007
8 Rau *et al.*, 2000
9 Lu, 2008
10 Pandol *et al.*, 2007; Tsuji *et al.*, 1994
11 Altavilla *et al.*, 2003
12 Yu *et al.*, 2002
13 Lu, 2008
14 Luthen *et al.*, 1995
15 Guarner & Malagelada, 2003
16 van Minnen *et al.*, 2007; Reid *et al.*, 2004
17 Musenga *et al.*, 2007; Peran *et al.*, 2006
18 Peran *et al.*, 2007; Yadav *et al.*, 2007
19 Muftuoglu *et al.*, 2006
20 Sahin *et al.*, 2007

DETAILED MECHANISM-BASED INVESTIGATIONS OF THE EFFECTS OF PROPHYLACTIC PROBIOTICS ON OXIDATIVE STRESS ARE LARGELY LACKING

THIS STUDY INVESTIGATED THE EFFECTS OF PRETREATMENT WITH PROBIOTICS ON OXIDATIVE STRESS DURING THE EARLY PHASE OF ACUTE PANCREATITIS

incidence of intestinal ischemia and necrosis[21], suggesting that probiotics may have been causative of an extra oxidative assault rather than ameliorative of oxidative damage. These results unfortunately demonstrate that detailed mechanism-based investigations of the effects of prophylactic multispecies probiotics on oxidative stress are largely lacking. This study therefore investigated the effects of pretreatment with probiotics on oxidative stress, acinar cell injury and pro-inflammatory markers during the early phase of AP. As the current concept of the pathophysiology of severe AP resembles that of major abdominal trauma, sepsis and other critical illnesses, the results of this experiment may shed further light on mechanisms of action behind positive effects of probiotics in preventive applications, such as elective abdominal surgery[22].

21 Besselink *et al.*, 2008
22 Sugawara *et al.*, 2006

MATERIALS AND METHODS

RATS

Male specific pathogen-free Sprague-Dawley rats (250-350 grams, B&K, Sollentuna, Sweden) were maintained under constant housing conditions (temperature (22°C), relative humidity (60%) and a 12-hour light/dark cycle), had free access to water and standard rat chow throughout the experiment. The experimental design (*figure 1*) was approved by the local committee of animal ethics. Fifty-three rats were randomly allocated into five groups: 1) non-operated control animals (n=5), 2) sham-procedure (n=12), 3) AP (n=12), 4) AP and placebo (n=12), 5) AP and probiotics (n=12).

SURGICAL PROCEDURES

At the start of the experiment, under general anesthesia using a combination of 2% Isoflurane gas (flow: 0.5 l/min O_2, 1.5 l/min air), a permanent gastric cannula was fitted in all rats as described previously[23], except for non-operated control rats. Animals in the probiotics and placebo groups were allowed to recover for four days, prior to the start of daily probiotics or placebo administrations.

The study product (*Ecologic® 641,* Winclove Bio Industries, Amsterdam, the Netherlands) consisted of six viable and freeze-dried strains: *Lactobacillus acidophilus* (W70), *Lactobacillus casei* (W56), *Lactobacillus salivarius* (W24), *Lactococcus lactis* (W58), *Bifidobacterium bifidum* (W23), and *Bifidobacterium lactis* (W52) (previously classified as *Bifidobacterium infantis*) and cornstarch and maltodextran as carrier substances. Placebo, (the same substance without bacteria) was packed in identical sachets and coded by the producer to guarantee blinding. Directly before administration, products were reconstituted in sterile water, for 15 minutes at 37°C. A single probiotic dose of 1.0 ml contained a total of 5 x 10^9 colony forming units (CFU) of bacteria. Probiotics or placebo were daily administered through the gastric cannula, starting five days before induction of AP. The cannula was flushed with 1.0 ml water after administration of probiotics or placebo to ensure gastric delivery of the product. Animals in the sham and AP group were daily administered 1.0 ml water through the gastric cannula.

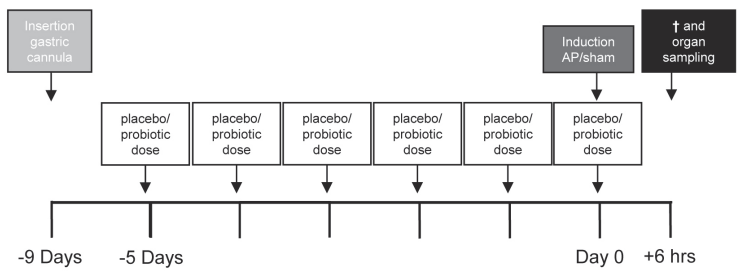
AP was induced as originally described by Schmidt *et al.* (1992) and previously performed by our group[24]. Pressure controlled retrograde infusion of 0.5 ml sterilized glycodeoxycholic acid (Sigma-Aldrich Chemie BV Zwijndrecht, the Netherlands) into the common bile duct was followed by intravenous infusion of cerulein (5µg/kg/h, 1ml/h, for 6 h, Sigma-Aldrich). No animals needed to be excluded for infusion pressures exceeding 35 mmHg. During the sham-procedure, the papilla of Vater was cannulated, but no glycodeoxycholic acid was infused, followed by 6 hours of intravenous saline infusion (1ml/hr).

23 van Minnen *et al.*, 2007
24 van Minnen *et al.*, 2007

FIGURE 1

Experimental design

Nine days prior to induction of pancreatitis, a permanent gastric cannula was fitted into all animals, except for the non-operated control animals. Probiotics or placebo was administered intragastrically once daily to the animals allocated into the probiotics and placebo group, starting 5 days prior to induction of acute pancreatitis. Nine days after the start of the experiment, acute pancreatitis (AP) was induced or a sham procedure was performed. After cerulein infusion, all animals were anesthetized for removal of organ samples.



COLLECTION AND PRESERVATION OF SAMPLES

After infusion of cerulein or saline, rats were anaesthetized by isoflurane inhalation. Whole blood was collected via cardiac puncture into EDTA coated tubes. A portion of the pancreatic tail was frozen *in situ* using a pre-frozen forceps, freeze-dried and stored at -80°C for analysis of ATP levels. Samples from the central part of the pancreas were fixed in neutral 4% buffered formalin, then washed in PBS containing 6.8% sucrose, dehydrated in acetone, embedded in Technovit 8100-plastic (Hereus-Kultzer, Wehrheim, Germany), and sectioned for histological analysis. For terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL), formalin fixed pancreas samples were embedded in optimum cutting temperature (OCT) compound (Histolab, Västra Frölunda, Sweden), and frozen at -80°C until sectioned. Remaining part of the pancreas was snap frozen in liquid nitrogen, stored at -80°C and freeze-dried within one week. Biochemical analyses, run in duplicates, were performed in freeze-dried tissues.

HISTOLOGICAL GRADING

Pancreatic tissue sections (2 μm) were hematoxylin-eosin (H&E) stained, coded and examined by a pathologist blinded to the experimental design. Severity of pancreatitis was graded according to the previously described[25], modified Spormann's (1989) scoring criteria (table 1). Infiltrating neutrophils were counted in four

25 Sandstrom *et al.*, 2005

random fields per slide. Necrosis was defined as loss of the acinar cell structure, including zymogen degranulation, loss of the basal basophilic/apical acidophilic staining of the cytoplasm, a pyknotic nucleus, and rupture of the cell membrane. At a 100x magnification, a minimum of 1000 random acinar cells per histological section were counted. The amount of acinar cell necrosis was related to the total area of pancreatic parenchyma and presented as a percentage of the total.

AMYLASE ACTIVITY

Amylase activity in plasma (U/l) was measured by routine methods in our clinical chemistry laboratory, using a two-step specific pancreas amylase assay (Diasys Diagnostic Systems International, Holzheim, Germany). After inhibition of salivary amylase the substrate 4.6-etylidene-(G7)-1-4-nitrophenyl-(G1)-α-D-maltoheptaoside (EPS) was cleaved by pancreas associated amylase in the sample, releasing 4-nitrophenylated oligosaccharides. A second substrate was added (α-glucosidase), resulting in release of p-nitrophenyl, which was measured as an increase in absorbance at 410 nm using a Advia 1800 autoanalyzer (Siemens AG, Munich, Germany).

PANCREATIC WATER CONTENT

To estimate pancreatic oedema, frozen tissue was weighed on a balance with readability of 0.01 mg (Mettler AT 250, American Instrument Exchange, Inc, Haverhill, MA) before and after freeze-drying. Results are expressed as percentages water of total tissue weight.

APOPTOSIS

Apoptotic cells were detected, using the 'in-situ cell death detection kit' (Roche Diagnostics, Bromma, Sweden). Pancreas sections (5 μm) were incubated in TUNEL reaction mixture for one hour at 37°C and counterstained with 0.5 μM 4·,6-diamidineo-2-phenylindole (DAPI). Slides were mounted in antifading Fluorescent Mounting Medium (DakoCytomation, CA, USA) and examined using confocal laser scanning microscopy (Sarastro 2000, Molecular Dynamics, Sunnyvale, CA, 60x oil immersion objective). For each test, negative controls were included. Apoptotic rate was determined by counting the number of TUNEL positive cells per 100 cells in 20 random photomicrographs in 4 tissue sections of 4 rats in each group.

Further quantification of apoptosis was performed by histone-associated DNA-fragmentation analysis in 50 μg pancreatic tissue, using the Cell Death Detection ELISA ^{PLUS} Kit (Boehringer Mannheim, Mannheim, Germany) as previously described[26]. Results were normalized to protein content as measured according to Bradford *et al.* (1976) in the original homogenates.

Caspase-3 activity was assessed in pancreatic tissue extracts, corresponding with 100 μg protein content. Freeze-dried tissue (5 μg) was homogenized at 4°C in 500 μl Reporter Gene Assay Lysis Buffer (Roche Diagnostics, Bromma, Sweden) as performed previously

26 Trulsson *et al.*, 2004

TABLE 1

Histological scoring of pancreas injury

Score	
Oedema	
0	None
1	Minimal oedema, expanded interlobular septa
2	Moderate oedema, expanded intralobular septa
3	Severe oedema, separated individual acini
Fat inflammation	
0	None
1	Minimal (<20 inflammatory cells per intermediate-power field (IPF) at ×200 magnification)
2	Moderate (20-50 inflammatory cells per IPF)
3	Severe (>50 inflammatory cells per IPF)
Parenchymal Inflammation	
0	None
1	Minimal (<20 inflammatory cells per intermediate-power field (IPF) at ×200 magnification)
2	Moderate (20-50 inflammatory cells per IPF)
3	Severe (>50 inflammatory cells per IPF)
Peripheral Necrosis	
0	None
1	Moderate
2	Severe
Fat Necrosis	
0	None
3	Focal necrosis, <5% of the total area of fat
5	and/or sublobular necrosis, <20% of the total area of fat
7	and/or lobular necrosis, >20% of the total area of fat
Parenchymal Necrosis	
0	None
3	Focal necrosis, <5% of the total area of parenchyma
5	and/or sublobular necrosis, <20% of the total area of parenchym
7	and/or lobular necrosis, >20% of the total area of parenchyma

by our group[27]. In short, the homogenate was centrifuged at 15,000 *g* for 30 minutes. The supernatant was used for the caspase activity assay and protein content determination. Equal amounts of protein were assayed in duplicates for each sample. Cleavage of the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC, PharMingen, Becton-Dickinson, Stockholm) by active caspase-3 was measured at an excitation wavelength of 380 nm and emission wavelength of 435 nm, using a fluorescence spectrophotometer (Varian Cary Eclipse Instrument, Palo Alto, CA, USA). Results are expressed as fluorescence units/ mg protein content.

ATP LEVELS

For maximal stability, cellular ATP was extracted in 1 M perchloric acid containing 1mM EDTA, for 30 minutes on ice. After centrifugation, 200 µl supernatant was neutralized by adding 100 µl 2.2 M $KHCO_3$. Samples were repeatedly mixed for 20 minutes on ice and subsequently centrifuged for 2 minutes at 6,000 *g* at 4°C. Cellular ATP levels were determined using a commercially available assay (ATP Determination Kit, A22066, Molecular Probes, Eugene, Oregon, USA) according to manufacturer's instructions. Light formed from ATP and luciferin in the presence of luciferase was measured at 560 nm (Multilabel Counter Victor$^3{}_{TM}$ V, PerkinElmer, Väsby, Sweden). ATP concentrations, expressed in µM per mg protein, were calculated using the included standard, diluted to the range of 0.5–10.0 µM.

PRO-INFLAMMATORY MEDIATORS

To investigate the pro-inflammatory reaction, the activity of IL-1β converting enzyme (ICE), which converts IL-1β into its active form[28] and NF-κB activation were quantified.

ICE activity was assessed in tissue corresponding with 100 µg protein content. Preparation of the tissue was similar to the previously described method for caspase-3 activity analysis[29]. ICE activity was measured using the ICE specific substrate Ac-Tyr-Val-Ala-Asp-AMC (AcYVAD-AMC, Calbiochem Darmstadt, Germany). Fluorescence of enzymatically cleaved AMC was measured at an excitation wavelength of 380 nm and emission wavelength of 435 nm. Results are given in fluorescence units/mg protein content.

To determine NF-κB activation, subunit p65 of activated NF-κB/Rel was quantified in nuclear protein by the ELISA kit 'TransAM NF-κB Family Kits' (Active Motif North America, California, USA) as previously described by our group (Trulsson et al., 2004). Activity of NF-κB is expressed as the absorbance ($OD_{450\ nm}$) of subunit p65 per mg of nuclear protein.

LIPID PEROXIDATION

To assess oxidative-induced cellular damage to lipid membranes, lipid peroxidation was analyzed in pancreatic tissue. Pancreatic samples were homogenized in 20 mM PBS containing 5 mM butylated hydroxytoluene to prevent sample

27 Trulsson *et al.*, 2004
28 Kostura *et al.*, 1989
29 Trulsson *et al.*, 2004

oxidation. Malondialdehyde (MDA) concentration was determined in supernatants using a lipid peroxidation assay kit (LPO-586; Byoxitech, OXIS International, Portland, OR, USA) according to manufacturer's instructions. MDA levels, normalized to protein content of the original homogenate and are expressed as pmol/mg protein.

GLUTATHIONE ASSAY

To estimate the anti-oxidative capacity, reduced and oxidized GSH contents were determined in pancreatic tissue and plasma using a commercially available assay (Glutathione Assay Kit II, Merck Chemicals, Hull, United Kingdom) according to the protocol provided by the manufacturer. Briefly, pancreatic tissue (1 mg) was homogenized in 150 µl ice-cold lysis buffer and centrifuged at 10,000 *g* for 15 min at 4°C. After protein determination, supernatants and plasma aliquots were deproteinized with 5% metaphosphoric acid (Sigma-Aldrich Chemie BV) and 4 M triethanolamine (Sigma-Aldrich Chemie BV) and plasma samples were lyophilized. Samples were then mixed with the Assay Cocktail reagents. After incubation at 25°C for 25 min, absorbance was measured at 405 nm. GSH levels were calculated using a standard curve generated by standards provided by the manufacturer. GSH content was determined by subtracting the amount of GSSG from the total GSH content and GSH/GSSG ratios were calculated. Results are expressed in µmol/mg protein.

CYSTEINE

In pancreas and plasma total cysteine, the rate limiting precursor of GSH, was determined using the spectrophotometric method developed by Gaitonde (1967) and expressed as nmol/mg protein and as nmol/ml plasma, respectively. Briefly, samples were reduced in acidic medium with 10 mM dithiothreitol, and conjugates of ninhydrin were assayed spectrophotometrically at 560 nm.

GLUTAMATE-CYSTEINE-LIGASE

De novo synthesis of GSH in red blood cells (RBCs) and pancreas was analysed by quantification of GCL enzyme activity as previously described[30]. To yield hemolysates, erythrocytes were obtained from separate EDTA blood samples by centrifugation at 900 *g* for 3 minutes after washing 3 times with 5 volumes of cold isotonic NaCl solution. Erythrocytes were lysed by the addition of 1 volume of 50 mM Tris-HCl buffer (pH 7.4), containing 1 mM EDTA, and by sonication for 2×20 sec. The erythrocyte membranes were removed by centrifugation at 18,000 *g* for 40 minutes. Tissues were homogenized in 250 mM sucrose containing 20mM Tris, 1mM EDTA, 20 mM boric acid, 2 mM serine, pH 7.4. The cytosolic protein fraction from crude homogenates was obtained by centrifugation and was subsequently filtered through microcon-10 (Millipore) tubes to remove endogenous inhibitors and substrates for GCL. GCL activity was determined as the difference between GSH synthesis in unblocked and GSH synthesis in samples blocked with 200mM 5-sulfosalicylic acid dehydrate and expressed as mmol GSH/min/mg protein.

30 White *et al.*, 2003

STATISTICAL ANALYSIS

All data were assessed for normal distribution using Shapiro–Wilk's test. Parametric values are presented as mean (SEM). Statistical analysis was performed by ANOVA followed by Tukey's HSD test for multiple comparisons (SPSS 13.0 Inc, Chicago, Illinois, USA). Non-parametric values are given as median (25-75th inter-quartile range). Comparisons were done by Kruskal-Wallis when more than two groups were compared. Mann-Whitney U test was used to compare two groups. Spearman's rank correlation coefficients were computed for correlation analyses. Considering Bonferroni's correction, P values <0.01 were considered significant.

RESULTS

SAFETY OF ADMINISTRATION OF VIABLE PROBIOTICS

No rats receiving probiotics showed signs of diarrhea, weight loss or loss of appetite during probiotic treatment. During the 5 day pretreatment period, the increase in animal weight was equal in all groups[31]. Mortality due to AP did not occur.

PROBIOTICS AMELIORATED ACUTE PANCREATITIS-ASSOCIATED PANCREATIC DAMAGE

Pancreatitis-induced injury was characterized by extensive oedema, hemorrhage and infiltration of neutrophils (fig 2A). Pretreatment with probiotics markedly reduced the severity of pancreatic injury[32] (fig 2B). In the pancreatitis animals, light microscopy revealed areas of complete structural damage by necrosis involving 12% of the pancreatic parenchyma and the presence of infiltrating neutrophils, both of which were reduced after probiotic pretreatment (fig 2C, D). The histopathological score for oedema was reduced by pretreatment with probiotics[33]. This corroborated with the reduction in water content in the probiotic treated animals (fig 2E). Interestingly, pretreatment with probiotics did not affect the increase in plasma amylase despite the significant attenuation in the severity of pancreatitis (fig 2F).

PROBIOTICS REDUCED ACUTE PANCREATITIS-INDUCED APOPTOTIC CELL DEATH

Laser confocal microscopy of fluorescent TUNEL stained slides revealed that AP-induced apoptosis was confined to small peripheral areas of pancreatic parenchyma (fig 3A). Pretreatment with probiotics attenuated the AP-induced apoptosis, showing a 70% reduction of the apoptotic rate in pancreas ($P<0.01$) (fig 3B). To further quantify apoptosis, DNA-fragmentation and caspase-3 activity were determined. Elevation in histone-associated DNA-fragmentation in the AP group was prevented by pretreatment with probiotics (fig 3C). This was in keeping with the reduction in caspase-3 activity in probiotic pretreated animals (fig 3D).

PROBIOTICS REDUCED ACUTE PANCREATITIS-INDUCED ATP DEPLETION IN PANCREATIC TISSUE

Apoptosis is a highly regulated form of cell death, involving many ATP-dependent steps. Depletion of cellular ATP is known to cause switching of form of cell death from apoptosis to necrosis[34]. AP reduced ATP levels by 37%[35] six hours after induction of pancreatitis (fig 4A). Pretreatment with probiotics normalized the ATP levels. ATP levels

31 sham 22.3 (3.8) *vs.* AP 21.9 (3.9) *vs.* placebo 20.6 (2.8) *vs.* probiotics 20.5 (2.1) gr/5 days
32 probiotics 1.5 (0.88-3.25) points *vs.* placebo 5.5 (3-6.1); $P<0.01$
33 probiotics 1 (0.5-1.1) points *vs.* placebo 1.5 (1.3-2); $P<0.01$
34 Lemasters, 2005
35 sham 0.93 *vs.* AP 0.59 μM/mg protein; $P<0.001$

correlated inversely with the area of necrosis (r=-0.76) (fig 4B), which supports the hypothesis that ATP depletion is the major determinant for a necrotic form of cell death.

PROBIOTICS REDUCED ACUTE PANCREATITIS-INDUCED INFLAMMATORY MARKERS IN PANCREATIC TISSUE

To further investigate the effects of probiotics on AP-associated elevation of pro-inflammatory mediators we assessed activation of NF-κB by analysis of translocated p65 into the nucleus and IL-1β converting enzyme (ICE), which converts pro-IL-1β into its active form[36].

The induction of AP resulted in a ~3-fold elevation of activated ICE (fig 5A, $P<0.001$). Pretreatment with probiotics attenuated the AP-associated elevation of ICE activity in pancreas. In addition, the AP-induced increase in activated NF-κB was normalized by probiotics (fig 5B).

PROBIOTICS REDUCED ACUTE PANCREATITIS-INDUCED OXIDATIVE STRESS AND INCREASED GSH CONTENT IN THE PANCREAS

As the amount of generated ROS exceeds the cellular anti-oxidative capacity, ROS cause cellular damage in the form of oxidative degradation of lipid membranes, which can be measured as lipid peroxidation[37]. Therefore, to assess oxidative stress induced damage in the early phase of AP, lipid peroxidation was quantified. AP caused a marked increase in lipid peroxidation in the pancreas, which was normalized by pretreatment with probiotics (fig 6A). As this reduction in lipid peroxidation after probiotic pretreatment, may have resulted from either reduced amounts of ROS, or an enhanced anti-oxidative capacity, we quantified local oxidized glutathione (GSSG) and GSH. In all pancreatitis groups oxidized glutathione (GSSG) increased two-fold compared to sham or control animals (fig 6B) and administration of probiotics did not prevent this increase, suggesting that both probiotic and placebo pretreated animals encountered a similar amount of ROS during the early phase of AP. Interestingly, probiotic pretreatment did not only prevent AP-induced GSH depletion, it also increased pancreatic GSH levels, even compared with control and sham operated rats (fig 6C), suggesting an increase in anti-oxidative capacity after pretreatment with probiotics. As expected, there was an inverse correlation between pancreatic GSH content and lipid peroxidation (r=-0.70) (fig 6D). Furthermore, there was a strong positive correlation between lipid peroxidation and pancreatic injury (r=0.90) (fig 6E) and an inverse correlation between GSH content and histological pancreatic damage (r=-0.67) (fig 6F), which supports the hypothesis that intracellular GSH protects against oxidative stress-induced pancreatic injury. In addition, pancreatic ATP levels showed an inverse correlation with lipid peroxidation (r=-0.78) (fig 7A), as well as a positive correlation between ATP levels and pancreatic GSH content (r=0.70) (fig 7B).

36 Kostura *et al.*, 1989
37 Lu, 2008

PROBIOTICS INDUCE ENHANCED SYSTEMIC GLUTATHIONE LEVELS

To next determine whether the found local increase in GSH after probiotic pretreatment was associated with a change in systemic GSH, plasma GSH levels were analyzed. Systemically, probiotic pretreatment demonstrated an even more pronounced effect on GSH levels resulting in an almost 2 fold increase in plasma GSH compared to control animals (fig 8).

DE NOVO SYNTHESIS OF GLUTATHIONE

In search of the source of increased levels of GSH the possibility of enhanced GSH biosynthesis was explored. *De novo* synthesis of GSH is regulated by availability of cysteine and GCL enzyme activity[38]. Firstly, plasma cysteine levels were equal in all groups (fig 9A), in contrast to local levels in the pancreas which were reduced during AP, regardless of pretreatment (fig 9B), suggesting that cysteine availability did not play a major role in the enhanced levels of GSH. Secondly, GCL activity in red blood cells (fig 9C) and in pancreas (fig 9D) was most abundant in the probiotics group, indicating enhanced GSH biosynthesis in probiotic pretreated animals.

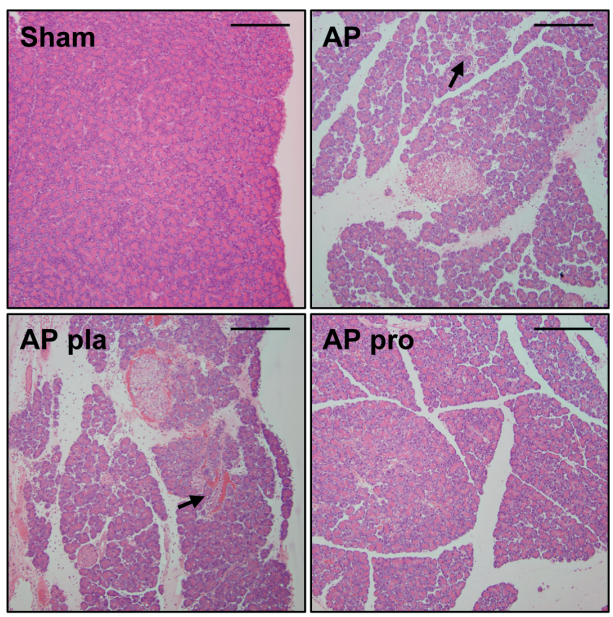
38 Lu, 2008

FIGURE 2

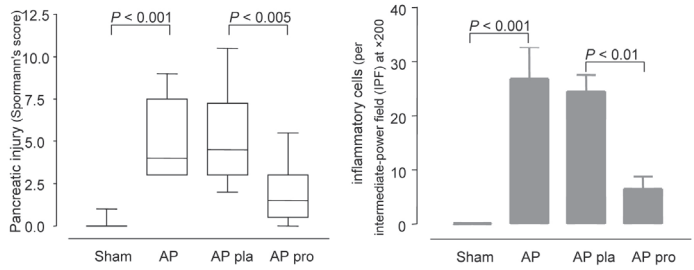
Probiotics reduced acute pancreatitis-associated tissue damage

After 5 days of pretreatment with placebo or probiotics, rats were subjected to acute pancreatitis, or a sham procedure (sham n=12; AP n=12; AP pla n=12; AP pro n=12). Sections of pancreas samples, obtained directly after completion of cerulein infusion or sham procedure, were stained with haematoxylin-eosin and graded according to modified criteria established by Spormann *et al.* (1989) (table 1). (A) Compared with sham operated animals, acute pancreatitis caused widespread oedema, inflammatory cell infiltration and focal necrosis (arrowhead). Placebo treated animals showed a severe degree of pancreatic damage with infiltration of inflammatory cells, oedema and hemorrhage (arrow head). Probiotic animals showed reduced inflammatory cell infiltration and were primarily characterized by a moderate degree of oedema. The pancreatic injury is typical of that seen in 4 sections from 12 rats per group. Bar = 200 μm. (B) Histopathological grading of pancreatic injury according to Spormann's criteria (1989). The graph shows the median (±range). Comparisons were performed using Kruskal-Wallis followed by Mann–Whitney *U* test. (C) Neutrophil count in 3-4 random high power fields per slide. The graph shows the average (±SEM). Comparisons were performed using ANOVA followed by Tukey's HSD. (D) Morphometric assessment of the extent of acinar cell necrosis (percentage of total pancreatic parenchyma). The graph shows the average (±SEM). Comparisons were performed using ANOVA followed by Tukey's HSD. (E) Pancreatic water content and plasma amylase (F) determined six hours after the induction of AP. The graph shows the average (±SEM). Comparisons were performed using ANOVA followed by Tukey's HSD.

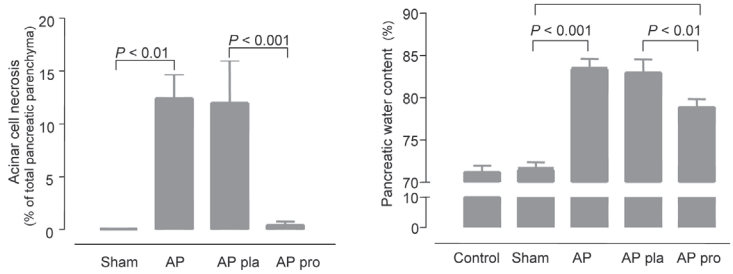
A



B / C


Pancreatic injury (Spormann's score)
12.5
10.0
7.5
5.0
2.5
0.0
P < 0.001
P < 0.005
Sham
AP
AP pla
AP pro
inflammatory cells (per intermediate-power field (IPF) at ×200
40
30
20
10
0
P < 0.001
P < 0.01
Sham
AP
AP pla
AP pro

D / E


Acinar cell necrosis (% of total pancreatic parenchyma)
20
15
10
5
0
P < 0.01
P < 0.001
Sham
AP
AP pla
AP pro
Pancreatic water content (%)
90
85
80
75
70
10
0
P <0.001
P < 0.001
P < 0.01
Control
Sham
AP
AP pla
AP pro

F

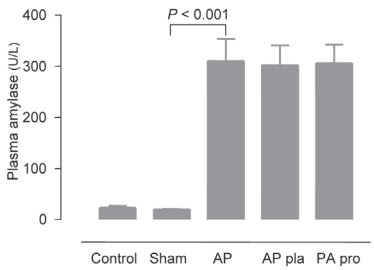
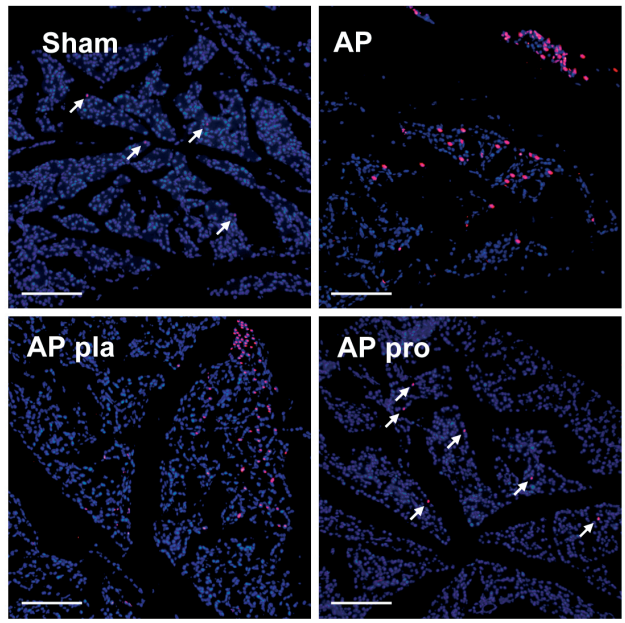

Plasma amylase (U/L)
400
300
200
100
0
P < 0.001
Control
Sham
AP
AP pla
PA pro

FIGURE 3

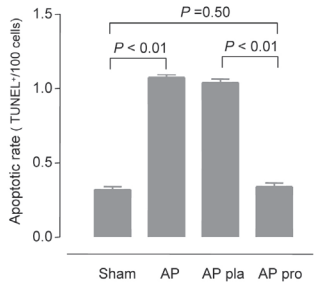
Probiotics reduced acute pancreatitis-associated apoptosis in the pancreas

After 5 days of pretreatment with placebo or probiotics, rats were subjected to acute pancreatitis, a sham procedure or were not operated upon (control n=5; sham n=12; AP n=12; AP pla n=12; AP pro n=12). Sections of pancreas, obtained after completion of cerulein infusion or sham procedure, were stained with fluorescent terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL, purple) and counterstained with 0.5 μM 4·,6-diamidineo-2-phenylindole (DAPI, blue). (A) Compared with sham operated animals, acute pancreatitis (AP) caused apoptosis confined to small peripheral areas of pancreatic parenchyma. Placebo treated animals (AP pla) also showed focal apoptosis in peripheral areas. Probiotic animals (AP pro) showed a reduction in apoptotic rate. The results shown are typical of those obtained from at least 4 rats per group. Bar = 100 μm (B) The graph shows the average (±SEM) number of TUNEL positive cells per 100 cells in 20 random photomicrographs of 4 sections per sample of at least 4 independently acquired samples from each group. Apoptosis was further quantified by determination of (C) DNA-fragmentation and (D) caspase-3 activation. The graphs show the average (±SEM). All analyses were run in duplicates. Comparisons were performed using ANOVA followed by Tukey's HSD.

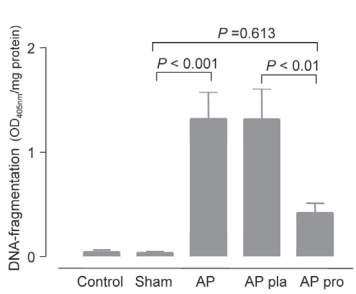
A



B


P =0.50
P < 0.01
P < 0.01
Apoptotic rate (TUNEL-/100 cells)
1.5
1.0
0.5
0.0
Sham
AP
AP pla
AP pro

C / D


P =0.613
P < 0.001
P < 0.01
DNA-fragmentation (OD405nm/mg protein)
2
1
0
Control
Sham
AP
AP pla
AP pro

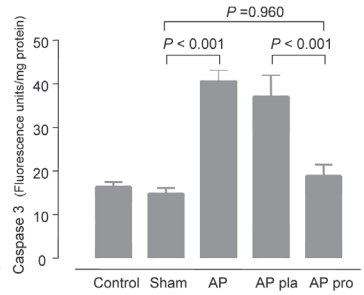

P =0.960
P < 0.001
P < 0.001
Caspase 3 (Fluorescence units/mg protein)
50
40
30
20
10
0
Control
Sham
AP
AP pla
AP pro

FIGURE 4

Probiotics attenuated acute pancreatitis-associated ATP depletion

After 5 days of pretreatment with placebo or probiotics, rats were subjected to acute pancreatitis, a sham procedure or were not operated upon (control n=5; sham n=12; AP n=12; AP pla n=12; AP pro n=12). (A) After completion of cerulein infusion or sham procedure, pancreas tissue samples were frozen *in situ*, freeze-dried and ATP levels were determined. The graph shows the median (±range). Comparisons were performed using Kruskal-Wallis followed by Mann–Whitney *U* test. (B) Correlation between the extent of acinar cell necrosis and pancreatic ATP levels was computed using Spearman's rank correlation coefficients.

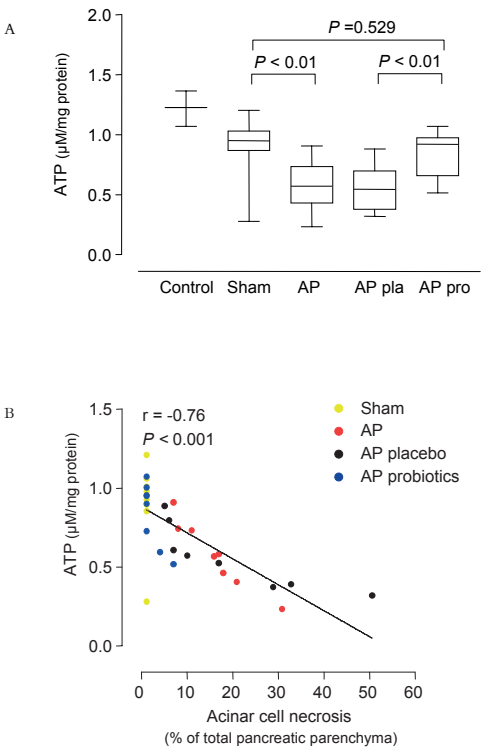


FIGURE 5

Probiotics reduced levels of acute pancreatitis-associated early inflammatory markers

After 5 days of pretreatment with placebo or probiotics, rats were subjected to acute pancreatitis, a sham procedure or were not operated upon (control n=5; sham n=12; AP n=12; AP pla n=12; AP pro n=12). After completion of cerulein infusion or sham procedure, inflammatory markers were assessed in pancreas tissue by quantification of (A) IL-1β converting enzyme (ICE) and (B) NF-κB levels. The graphs show the average (±SEM). All analyses were run in duplicates. Comparisons were performed using ANOVA followed by Tukey's HSD.

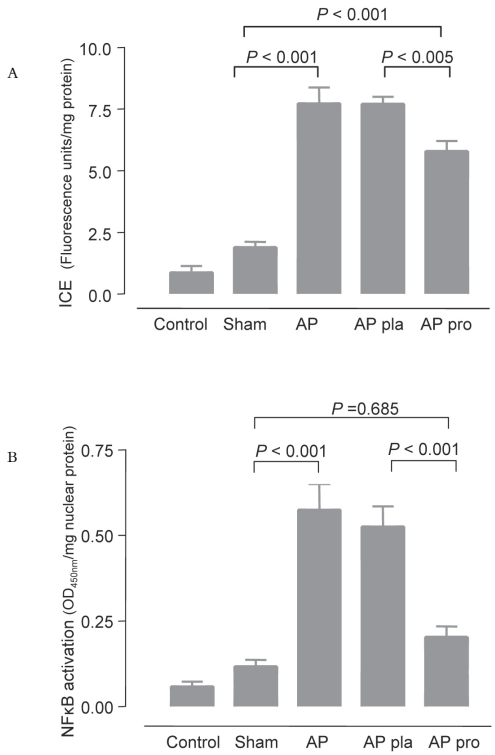


FIGURE 6

Probiotics reduced acute pancreatitis-induced oxidative stress in the pancreas

After 5 days of pretreatment with placebo or probiotics, rats were subjected to acute pancreatitis, a sham procedure or were not operated upon (control n=5; sham n=12; AP n=12; AP pla n=12; AP pro n=12). Anti-oxidant status was investigated in pancreas tissue, after cerulein infusion or sham procedure, by determination of (A) lipid peroxidation (MDA levels) (B) oxidized glutathione levels (GSSG) and (C) reduced glutathione levels (GSH). The graphs show the average (±SEM). All analyses were run in duplicates. Comparisons were performed using ANOVA followed by Tukey's HSD. Correlations between (D) lipid peroxidation and pancreatic GSH content, (E) pancreatic injury and lipid peroxidation and (F) pancreatic injury and GSH content were computed using Spearman's rank correlation coefficients.

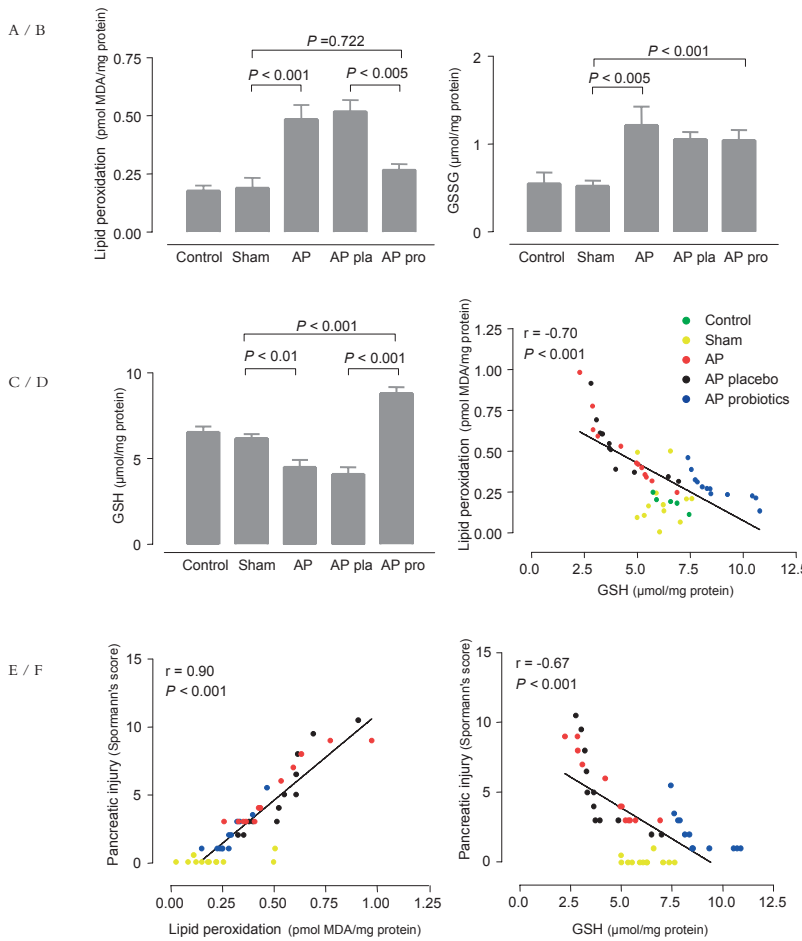


FIGURE 7

Correlation analysis between pancreatic ATP levels and local oxidative status

Correlations between (A) lipid peroxidation and pancreatic ATP levels and (B) local GSH levels and pancreatic ATP levels were computed using Spearman's rank correlation coefficients.

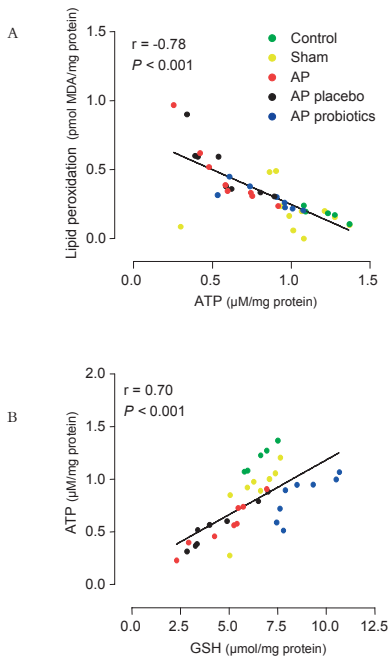


FIGURE 8

Probiotics induce enhanced systemic glutathione levels

After 5 days of pretreatment with placebo or probiotics, rats were subjected to acute pancreatitis, a sham procedure or were not operated upon (control n=5; sham n=12; AP n=12; AP pla n=12; AP pro n=12). After cerulein infusion or sham procedure, plasma glutathione levels were quantified. The graph shows the average (±SEM). Analyses were run in duplicates. Comparisons were performed using ANOVA followed by Tukey's HSD.

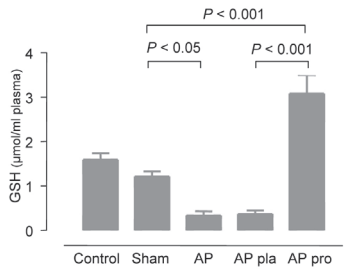
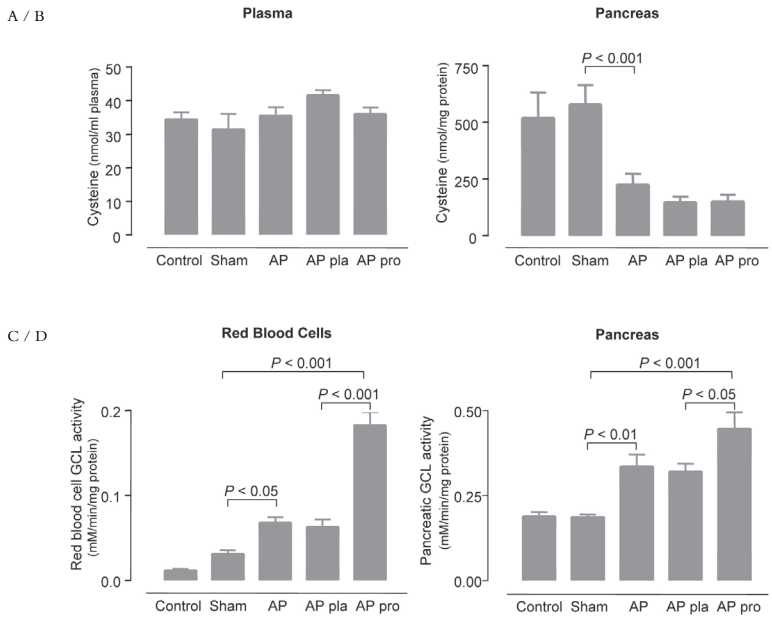


FIGURE 9

Probiotics have no effect on cysteine availability, but induce glutamate-cysteine-ligase activity

After 5 days of pretreatment with placebo (pla, n=12) or probiotics (pro, n=12), rats were subjected to acute pancreatitis (AP, n=12), a sham-procedure (n=12) or not operated (control, n=5). Six hours after induction of the AP or sham-procedure, cysteine availability in plasma (A) and pancreas (B) and glutamate-cysteine-ligase (GCL) activity in red blood cells (C) and pancreas (D) were determined. The graphs show average (±SEM). All analyses were run in duplicates. Comparisons were performed using ANOVA followed by Tukey's HSD.



DISCUSSION

The present study is the first to demonstrate that multispecies probiotics enhance *de novo* synthesis of GSH and increase GSH content both locally in the pancreas as well as systemically. After pretreatment with probiotics, oxidative stress, subsequent acinar cell injury and the local inflammatory response were ameliorated in a well established model of severe AP.

Oxidative stress is defined as a lack of sufficient anti-oxidative reserve during times of increased ROS production. There has been increasing awareness of the important role that oxidative stress plays in many inflammatory illnesses, including AP. In experimental as well as in clinical AP there is evidence of depletion of circulating anti-oxidant levels[39] with the degree of depletion corresponding to disease severity[40]. When produced under physiological conditions, ROS are captured by sufficient amounts of anti-oxidants. However, during the development of AP the increase in ROS exceeds the anti-oxidant capacity of endogenous defense systems[41] resulting in oxidative stress-induced damage[42]. Indeed, our data show that AP induced increased oxidative damage to lipid membranes, which was prevented by pretreatment with probiotics.

Besides lipid membranes, ROS are also capable of attacking other cellular targets resulting in cellular injury. Indeed, anti-oxidant therapy has been shown to ameliorate pancreatic injury in experimental AP[43]. In keeping with those findings our results show that pretreatment with probiotics attenuates the morphological severity and oedema formation in experimental AP. Moreover, the correlation between lipid peroxidation and pancreatic injury suggests that oxidative stress mediates the progression of AP.

In response to (oxidative) injury, acinar cells produce and release TNF-α, a pro-inflammatory cytokine which has shown to stimulate apoptosis in acinar cells[44]. In addition, ROS also directly induce apoptosis by damaging DNA. It is worth mentioning that an inverse correlation between acinar cell apoptosis and severity of AP has been reported. Indeed, extensive apoptotic acinar cell death was associated with a mild course of pancreatitis whereas sever AP is characterized by extensive necrosis[45]. This however is in apparent contrast with our current study, which demonstrates a concurrent reduction in severity and apoptosis in the probiotic pretreated group. While, our findings show that probiotics reduce DNA-fragmentation and caspase-3 activity in the pancreas, the maximum apoptotic rate of 1.1% suggests that apoptosis most likely plays a less significant role in the early phase of this model of AP. Similarly, Rau and colleagues (2001) studying the effects of ICE inhibition on cell death during severe AP, showed that apoptosis is not a relevant factor in the determination of severity during the early phase of experimental severe AP.

The development of pancreatic necrosis results in a dramatic increase of

39 Bonham *et al.*, 1999
40 Curran *et al.*, 2000
41 Luthen *et al.*, 1995
42 Tsuji *et al.*, 1994; Rau *et al.*, 2000
43 Esrefoglu *et al.*, 2006; Ohashi *et al.*, 2006
44 Gukovskaya *et al.*, 1997; Yu *et al.*, 2002
45 Bhatia, 2004

mortality in AP patients[46]. In AP, ROS cause severe mitochondrial dysfunction, resulting in ATP depletion. Apoptosis is an ATP-dependent process, which makes it impossible for the injured and ATP-depleted acinar cell to complete the apoptotic process. Under these conditions necrosis would be the only form of cell death possible[47]. In accordance with this hypothesis, the current study shows a concurrent amelioration of ATP-depletion and necrosis after pretreatment with probiotics. In addition, the hypothesis that oxidative stress causes mitochondrial damage and subsequent loss of ATP[48] is in keeping with the inverse correlation between pancreatic ATP levels and lipid peroxidation, found in our study.

Acinar cells respond to oxidative stress-induced injury with activation of NF-κB, causing a pro-inflammatory response[49]. Activation of cytokines such as TNF-α and IL-1β, have been shown to enhance local tissue destruction and to cause distant organ complications[50]. Our data show that pretreatment with probiotics, possibly mediated by a reduction of pancreatic oxidative stress, attenuates AP-induced NF-κB and ICE activity. ICE converts pro-IL-1β into its active form[51], which in turn has been shown to play a pivotal role in local and systemic complications in severe AP[52]. Moreover, both clinical and experimental studies have shown that ICE activity is an important determinant of severity of AP[53].

Of note, no difference in plasma amylase levels was observed, despite a marked amelioration of the severity of the AP after probiotic pretreatment. Although a direct role of GSH cannot be excluded, these results suggest that enhanced pancreatic GSH levels are associated with attenuated severity, without changing pathways of regulation of pancreatic protein secretion. Our findings are in accordance with data from Ethridge *et al.* (2002) demonstrating that in spite of an attenuation of severity of AP that was sorted by inhibition of cyclooxygenase-2, no differences in serum amylase were demonstrated. In addition, also in AP patients, only a weak association between amylase levels and severity of the pancreatitis can be demonstrated[54].

Taken together the above, our study demonstrates that pretreatment with probiotics putatively ameliorates the severity of AP via a reduction in oxidative stress-induced injury. This probiotic effect may have been due to either a reduction of ROS in the pancreas, or an enhanced defense mechanism against oxidative stress. GSH represents the major endogenous defense system against oxidative stress[55] and has been shown to be inversely correlated with the APACHE II score in patients with AP[56]. While scavenging ROS, GSH is oxidized to GSSG, being an indirect measure of the total amount of ROS, neutralized by GSH. In our study, probiotics showed no effect on pancreatic GSSG levels, indicating an equal cellular ROS production in placebo and probiotics pretreated rats. AP-induced GSH depletion, however was not only prevented in probiotic pretreated animals, probiotics increased pancreatic as well as systemic GSH

46 Frossard *et al.*, 2008
47 Fortunato *et al.*, 2006
48 Lemasters, 2005
49 Altavilla *et al.*, 2003; Yu *et al.*, 2002
50 Mayer *et al.*, 2000
51 Kostura *et al.*, 1989
52 Denham *et al.*, 1997
53 Rau *et al.*, 2001
54 Winslet *et al.*, 1992
55 Lu, 2008
56 Rahman *et al.*, 2004

levels even compared with control animals. Our data concur with recent data[57] showing that probiotics enhance GSH levels. Peran *et al.* (2006) showed in a model of rat colitis that the preventive effect of *Lactobacillus fermentum* was mediated by bacterial release of GSH, attenuating oxidative stress. However, this is the first time that probiotics have been shown to increase GSH in pancreatic tissue. Moreover, in the probiotic pretreated group, enhanced pancreatic GSH content may have maintained normal lipid peroxidation levels, by counteracting the large amount of pancreatic ROS[58]. The inverse correlation between pancreatic GSH content and lipid peroxidation, indicating oxidative stress-induced cellular injury and histological scoring of pancreatic injury emphasizes the importance of this endogenous defense mechanism.

Our results describe an increase in GSH biosynthesis after pretreatment with probiotics; demonstrated by an increase in GCL activity and increased local and systemic GSH levels. GCL activity is up-regulated under conditions where increased cellular defense is necessary, i.e. low dose pro-inflammatory stimuli as TNF-α or NF-κB or sublethal oxidative stress, resulting in increased cell resistance against subsequent and potential lethal insults[59].

In contrast to the results described herein, our group recently conducted a double blind, randomized trial in nearly 300 severe AP patients which demonstrated, contrary to any expectations, that administration of probiotics early after the onset of AP more than doubled the relative risk of mortality[60]. Taken together the above, a hypothetical model can be envisioned in which administration of probiotics in healthy subjects causes minor stress, inducing upregulation of anti-oxidative enzymes and consequently preconditioning for a large oxidative assault. Whereas, in critically ill AP patients, the same minor stressor may only further aggravate the critical condition. Consequently, probiotics may not be a treatment option in critically ill.

In addition, even though anti-oxidant therapy has been shown to ameliorate the severity of experimental AP[61], it should be noticed that pancreatic oxidative stress and excessive ROS generation are early features in AP and consequently not a good target for clinical therapy. This has recently been shown in a randomized controlled trial where the found results in human subjects were not that encouraging[62]. Probiotics however, possess a whole array of mechanisms of action [63]and to fully benefit from the anti-oxidative action of probiotics, preventive applications need to be thought of. For instance in elective major abdominal surgery, in which intestinal oxidative stress is to be expected [64] pretreatment with probiotics has already shown promising results in clinical trials[65]. In addition, post endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis is one of the major complications that has plagued this endoscopic procedure and occurs at a relatively constant rate of 10%[66]. The elective nature of the procedure gives a unique opportunity for early modification of the disease and perhaps prophylaxis.

57 Yadav *et al.*, 2007; Peran *et al.*, 2007
58 Lu, 2008
59 Lu, 2008
60 Besselink *et al.*, 2008
61 Yu *et al.*, 2002; Altavilla *et al.*, 2003
62 Siriwardena *et al.*, 2007
63 Guarner & Malagelada, 2003
64 Thomas *et al.*, 2003
65 Rayes *et al.*, 2007; Sugawara *et al.*, 2006
66 Andriulli *et al.*, 2007

THE FIRST TIME THAT PROBIOTICS HAVE BEEN SHOWN TO INCREASE GLUTATHIONE IN PANCREATIC TISSUE

PROBIOTICS ENHANCE GLUTATHIONE BIOSYNTHESIS, INCREASE GLUTATHIONE CONTENT AND REDUCE OXIDATIVE STRESS IN THE PANCREAS

As the used product is a multispecies combination of probiotic strains, it is worth noting that, the found effects depend on the specific combination of the applied bacteria. Additional studies will be necessary to elucidate the effects of the separate strains as well as a possible synergistic effect of this specific combination of probiotics. In addition, probiotic effects are mostly strain specific and cannot be generalized for other probiotic combinations.

Overall, our findings show that administration of multispecies probiotics enhances GSH biosynthesis both systemically and locally and consequently increases GSH content in the pancreas and reduces oxidative stress in acinar cells resulting in prevention of pancreatic peroxidative damage and amelioration of the local inflammatory response, finally reducing the severity of experimental AP. Our data do not only demonstrate the ability of probiotics to augment the anti-oxidative defense, but also underscore the importance of oxidative stress and the endogenous anti-oxidant defense mechanisms during the early phase of AP.

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Probiotics reduce acute pancreatitis-induced liver injury and enhance anti-oxidant capacity via glutamate-cysteine-ligase induction.

Probiotics enhance hepatic GSH synthesis

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Submitted for publication

ABSTRACT

BACKGROUND

During acute pancreatitis (AP), the liver is one of the affected organs as well as an amplifier of the inflammatory process. Many factors are likely to cause liver injury, including oxidative stress. Probiotics have shown to reduce oxidative stress and liver injury in hepatic ischemia-reperfusion experiments. The objective was to study effects of probiotics on oxidative stress, liver injury and pro-inflammatory markers in the early phase of AP.

METHODS

53 Spraque-Dawley rats were allocated into: 1) controls, non-operated, 2) sham-operated, 3) AP, 4) AP + probiotics (Ecologic®641) and 5) AP + placebo. Pancreatitis was induced by intraductal glycodeoxycholate infusion and intravenous cerulein (6hrs). Daily probiotics or placebo were administered intragastrically, starting five days prior to induction of AP. After cerulein infusion, liver samples were collected for analysis of lipid peroxidation, glutathione and glutamate-cysteine-ligase levels, histological scoring and NF-κB activity.

RESULTS

AP caused increased hepatic oxidative damage[1], which correlated with the pro-inflammatory marker:

1 lipid peroxidation: AP 0.214 *vs.* sham 0.079 pmol MDA/mg protein; $P<0.001$

NF-κB (r=0.85). Probiotics attenuated AP-induced NF-κB activity[2], lipid peroxidation[3] and liver injury. Interestingly, not only was AP-induced decrease in GSH prevented after probiotics[4]. Probiotics increased GSH, even compared with sham rats[5]. Hepatic biosynthesis of glutathione (glutamate-cysteine-ligase activity) was enhanced in probiotic pretreated animals.

CONCLUSIONS

Probiotics reduced peroxidative liver injury and subsequent inflammatory response during experimental AP. The mechanisms of this protective effect may include upregulation of hepatic glutathione biosynthesis.

2 probiotics 0.062 *vs.* placebo 0.098 OD_{450nm}/mg nuclear protein, $P<0.05$
3 probiotics 0.091 *vs.* placebo 0.199 pmol MDA/mg protein; $P<0.001$
4 probiotics 16.01 *vs.* placebo 9.01 μmol/mg protein, $P<0.001$
5 probiotics 16.01 *vs.* sham 12.10 μmol/mg protein, $P<0.05$

INTRODUCTION

The clinical manifestation of acute pancreatitis (AP) can range over a continuum from local peripancreatic inflammation to a widespread systemic inflammatory response with distant organ injury[6]. Although the exact pathophysiological factors are unknown, there are indications that oxidative stress may be an important determinant of disease severity[7].

Early in the course of AP, extensive release of reactive oxygen species (ROS) overwhelms the anti-oxidative capacity of acinar cells. Indeed, Rau *et al.* (2000) have shown that as early as 5 minutes after induction of AP, the imbalance between ROS and anti-oxidant systems causes pancreatic oxidative damage. The involvement of ROS in the course of AP is however not restricted to pancreatic tissue. Oxidative stress appears also to be involved in systemic manifestations of the disease[8].

The liver is the first organ exposed to and suffering from pancreatic factors released from the pancreas before dilution by the systemic circulation. Many pancreatic factors are likely to cause liver injury, including ROS. As hepatic injury occurs, Kupffer cell activation takes place, resulting in release of pro-inflammatory mediators, including nuclear factor (NF)-κB. This attracts neutrophils that secrete ROS, aggravating oxidative damage of the liver. In this regard the liver cannot only be considered a sentinel target organ, but also as an amplifier of the inflammatory process, contributing to further remote organ injury[9].

Another important cause for development of liver injury during AP is the status of the gastrointestinal tract. Oxidative damage causes intestinal mucosal barrier failure, allowing luminal content (e.g. bacteria) to translocate to the liver *via* the portal vein[10].

All cells are equipped with an endogenous anti-oxidant defense, in which glutathione (GSH) plays a key role. Intracellular GSH levels are predominately determined by biosynthesis which is mainly controlled by the activity of the rate-limiting enzyme glutamate-cysteine-ligase (GCL). In response to sublethal oxidative stress, enhanced gene expression of GCL occurs, increasing GSH levels and thereby improving resistance towards subsequent potential lethal oxidative insults[11].

The concept that probiotics can have anti-oxidative properties is relatively new and unexplored. Xing *et al.* (2006) showed that pretreatment with probiotics reduced oxidative stress and liver injury in experimental hepatic ischemia-reperfusion. We have previously demonstrated that pretreatment with probiotics reduced severity of experimental AP[12] and decreased intestinal barrier failure through induction of GSH biosynthesis[13]. Since both pancreatic damage and intestinal barrier failure contribute to hepatic injury in AP, we investigated the effects of probiotic pretreatment on the severity of oxidative stress, liver injury, and subsequent inflammatory response in experimental AP.

6 Frossard *et al.*, 2008
7 Abu-Zidan *et al.*, 2000; Rau *et al.*, 2000
8 Folch *et al.*, 1998
9 Folch-Puy, 2007
10 Lutgendorff *et al.*, 2009
11 Lu, 2008
12 Lutgendorff *et al.*, 2008
13 Lutgendorff *et al.*, 2009

THE LIVER CANNOT ONLY BE CONSIDERED A SENTINEL TARGET ORGAN, BUT ALSO AN AMPLIFIER OF THE INFLAMMATORY PROCESS, CONTRIBUTING TO FURTHER REMOTE ORGAN INJURY

WE INVESTIGATED THE EFFECTS OF PROBIOTIC PRETREATMENT ON OXIDATIVE STRESS, LIVER INJURY AND SUBSEQUENT INFLAMMATORY RESPONSE IN ACUTE PANCREATITIS

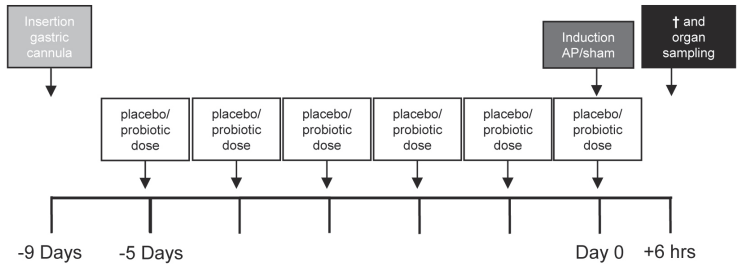
MATERIALS AND METHODS

RATS

Fifty-three male Sprague-Dawley rats (B&K, Sollentuna, Sweden, 250-350gr) were randomly allocated into five groups: 1) non-operated controls (n=5), 2) sham procedure (n=12), 3) AP (n=12), 4) AP and placebo (n=12) and 5) AP and probiotics (n=12). The experimental design (fig 1) was approved by the local committee of animal ethics.

FIGURE 1 **Experimental design**

Nine days prior to induction of pancreatitis, a gastric cannula was fitted into all animals, through which probiotics or placebo was administered intragastrically once daily, starting 5 days prior to induction of AP. After cerulein infusion, all animals were anesthetized for removal of organ samples.



PROBIOTICS AND PLACEBO

Probiotics (*Ecologic® 641*, Winclove Bio Industries, Amsterdam, The Netherlands) consisted of viable, freeze-dried probiotic strains; *Lactobacillus acidophilus* (W70), *Lactobacillus casei* (W56), *Lactobacillus salivarius* (W24), *Lactococcus lactis* (W58), *Bifidobacterium bifidum* (W23), and *Bifidobacterium lactis* (W52). Placebo consisted of carrier substance only (corn-starch + maltodextrin). Before administration of the doses, products were reconstituted in sterile water, for 15 minutes at 37°C. Single probiotics dose volumes of 1.0ml contained $5x10^9$ CFU bacteria. Placebo or probiotics were administered intragastrically through a permanent gastric cannula once daily, starting five days prior to induction of AP.

SURGICAL PROCEDURES

All surgical procedures were performed under general anesthesia (2% isoflurane). A gastric cannula was fitted in all rats as previously described[14], except for non-operated control rats. Animals in the probiotics and placebo groups were allowed to recover for four days, prior to start of daily probiotics or placebo administrations.

Five days after the start of treatments, AP was induced as described by Schmidt *et al.* (1992). Briefly, retrograde infusion of 0.5ml glycodeoxycholic acid (10mM, Sigma-Aldrich Chemie BV Zwijndrecht, The Netherlands) into the common bile duct was followed by intravenous infusion of cerulein (5μg/kg/hr for six hours). Sham-procedures were conducted as previously described[15].

After cerulein infusion, rats were anaesthetized by isoflurane inhalation and blood samples were collected via cardiac puncture into EDTA coated tubes. Portion of the right liver lobe was fixed in formalin (4%) over night, embedded in Technovit 8100-plastic (Hereus-Kultzer, Wehrheim, Germany) and sectioned. The remaining part of the right lobe of the liver was excised, snap frozen in liquid nitrogen and stored at -80°C for biochemical analyses, which were run in duplicates. Parts of ileum and pancreatic tail were sampled and processed as previously described[16].

INTESTINAL BARRIER FUNCTION

Mucosal permeability to ^{51}Cr-EDTA, horseradish peroxidase and *Escherichia coli* was determined in Ussing chambers as previously described[17].

HISTOLOGICAL GRADING

Liver sections (2μm) were hematoxylin-eosin (H&E) stained and coded before examination by a pathologist blinded to the experimental design. Liver injury was graded using a modification of Calabrese's criteria[18], scoring sinusoidal stasis (0-1), vacuolization (0-3) hepatocyte necrosis (0-1) and leucocyte infiltration (0-1). Necrotic areas were identified by visual microscopic scoring. Manual outlining and quantification of necrotic areas was performed with the aid of an image analysis software program (Saisam V.4.2.2.; Microvision Instruments, Evry Cedex, France) and expressed as percentages of whole surface area. Intestinal mucosal damage was scored in ileal H&E stained sections according to criteria by Chiu *et al.* (1970).

AP was confirmed by histological analysis of H&E stained pancreatic tail sections, using Spormann's criteria[19].

LIVER INJURY

Plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as markers of liver injury. Plasma levels of AST and ALT were measured on an automated analyser (Olympus AU 600, Diamond Diagnostic, Holliston, MA, USA).

14 Lutgendorff *et al.*, 2008
15 Lutgendorff *et al.*, 2008
16 Lutgendorff *et al.*, 2009
17 Lutgendorff *et al.*, 2009
18 Calabrese *et al.*, 1997
19 Spormann *et al.*, 1989

QUANTIFICATION OF APOPTOSIS

In order to quantify the degree of apoptosis, DNA-fragmentation was determined in liver cell suspensions (Cell Death Detection ELISA-^{PLUS} Kit Boehringer-Mannheim, Germany) as described previously[20]. Results are normalized to protein content, determined according to Bradford's method[21] and expressed as ratios to control animals.

Caspase-3 activity was chosen as second marker of apoptosis and assessed as described in previous publications[22]. Results are expressed as fluorescence units/mg protein content.

PRO-INFLAMMATORY MEDIATORS

To determine the activation of pro-inflammatory mediators in the liver, IL-1β-converting-enzyme (ICE) and NF-κB-activation were assessed as previously described[23]. Results are given in fluorescence units/mg protein and absorbance (OD_{450nm}) of subunit p65/mg of nuclear protein, respectively.

LIPID PEROXIDATION

Lipid peroxidation was quantified to determine the extent of oxidative damage induced by ROS. Malondialdehyde (MDA) concentration was determined using a lipid peroxidation assay kit (LPO-586; Byoxitech, OXIS International, Portland, OR, USA), according to manufacturer's instructions. MDA levels are expressed as pmol/mg protein.

GLUTATHIONE ASSAY

Oxidative damage is the result of ROS that could not be captivated by endogenous anti-oxidants. The extent of oxidative damage is therefore depended on two factors: the level of ROS and the capacity of the endogenous anti-oxidative defense system[24]. To estimate the anti-oxidative capacity, GSH, the most important endogenous anti-oxidant was quantified. Oxidized GSH (GSSG) is the resultant of ROS scavenged by GSH and is therefore an indirect measure of levels of ROS. Reduced and oxidized GSH levels were determined as previously described[25] and expressed as nmol/mg protein.

CYSTEINE

Biosynthesis of GSH is dependent on the availability of its precursors: glutamate and cysteine, of which the latter is rate-limiting[26]. Cysteine levels were determined

20 Lutgendorff *et al.*, 2008
21 Bradford, 1976
22 Lutgendorff *et al.*, 2008; Lutgendorff *et al.*, 2009
23 Lutgendorff *et al.*, 2008
24 Lu, 2008
25 Lutgendorff *et al.*, 2009
26 Lu, 2008

using the spectrophotometric method developed by Gaitonde[27] and expressed as nmol/mg protein. Briefly, liver samples were reduced in acidic medium with 10mM dithiothreitol, and conjugates of ninhydrin were assayed spectrophotometrically at 560nm.

GLUTAMATE-CYSTEINE-LIGASE

De novo synthesis of GSH is furthermore determined by GCL-activity, which catalyzes the chemical reaction leading to GSH formation. GCL enzyme activity was analyzed as previously described[28]. Hepatic tissue was homogenized in 250 mM sucrose containing 20 mM Tris, 1 mM EDTA, 20 mM boric acid, 2 mM serine, pH 7.4 and centrifuged for 10 min at 10,000xg. GCL-activity was determined in cytosolic protein fraction as the difference between GSH synthesis in unblocked and GSH synthesis in samples blocked with 200mM 5-sulfosalicylic acid dehydrate and expressed as mmol GSH/min/mg protein.

STATISTICAL ANALYSIS

Parametric values are presented as mean (SEM) and analysis of variance (ANOVA) was followed by Tukey's HSD. Non-parametric values are given as median (25–75th interquartile range). Comparisons between two groups were made by Mann-Whitney U test and between multiple groups by Kruskal-Wallis (SPSS 13.0 Inc, Chicago, Illinois, USA). For the analysis of associations between hepatic damage and pancreatic factors as well as, intestinal barrier dysfunction, data from previous publications from our group[29] were used to compute Spearman's rank correlation coefficients. XYZ-plots were created using Origin 8.0 (OriginLab Co., Northampton, MA). $P<0.01$ was considered statistically significant.

27 Gaitonde, 1967
28 Lutgendorff *et al.*, 2009
29 Lutgendorff *et al.*, 2008 and Lutgendorff *et al.*, 2009 respectively

RESULTS

ACUTE PANCREATITIS CAUSED OXIDATIVE-INDUCED LIVER DAMAGE

Severe AP was confirmed by histological scoring of pancreatic injury[30]. Mortality due to AP did not occur.

To confirm the hypothesis that ROS cause cytotoxic injury during early phase AP, lipid peroxidation was quantified as marker of oxidative stress-induced damage. Indeed, AP caused 40% increase in lipid peroxidation (fig 2A). This finding coincided with AP-induced increase in plasma markers for hepatic injury, i.e. AST and ALT (fig 2B, C).

Furthermore, histological examination revealed extensive AP-induced liver injury, showing in hepatocellular vacuolization and necrosis. Inflammatory infiltration was predominant in necrotic areas (fig 2D). Histological scoring demonstrated a significant increase in liver damage compared to sham operated rats (fig 2E) and a high degree of necrosis after induction of pancreatitis (20% (4.3), fig 2F).

Besides necrosis, apoptosis is a common feature of liver injury caused by an oxidative insult and was assessed by quantification of apoptotic markers i.e. DNA-fragmentation and caspase-3. AP caused a 2-fold increase in DNA-fragmentation (fig 3A). This corroborated with enhanced caspase-3 activity (fig 3B).

To further investigate the pro-inflammatory response subsequent to hepatic damage, levels of inflammatory markers, i.e. interleukin-1β-converting-enzyme 1 (ICE), which converts pro-interleukin-1β into its active form[31] and NF-κB were assessed and found to be markedly elevated after the induction of AP (fig 4A, B). Both markers correlated with oxidative damage (lipid peroxidation) (fig 4C, D).

Both pancreatic injury[32] and intestinal barrier dysfunction[33] are known to be contributing factors to hepatic injury in the course of AP. To assess these pathophysiological factors, parameters of intestinal and pancreatic damage were correlated to AP-induced liver injury and hepatic release of pro-inflammatory mediators (table 1). Interestingly, factors indicating intestinal barrier dysfunction or mucosal damage correlated better with liver injury and the subsequent hepatic pro-inflammatory response as compared with parameters of pancreatic damage[34]. For example, correlation analysis between liver injury, pancreatic lipid peroxidation (fig 5A) and liver injury and ileal lipid peroxidation (fig 5C) demonstrated that ileal oxidative damage (lipid peroxidation) correlates better with liver injury as compared to pancreatic lipid peroxidation. This also showed in the 3D XYZ-plot, in which more extensive intestinal oxidative damage correlated with the severity of liver damage. In contrast to pancreatic lipid peroxidation; this did not seem to correlate very well with hepatic injury (fig 5B).

30 0 (0-0) vs. 3 (2-5.1) after induction of pancreatitis; P<0.001
31 Kostura *et al.*, 1989
32 Folch-Puy, 2007
33 Wang *et al.*, 2009
34 average correlation coefficient of 0.82 (0.02) vs. pancreas 0.5 (0.04), P<0.001

PROBIOTICS ATTENUATED ACUTE PANCREATITIS-INDUCED LIVER INJURY

Rats receiving probiotics showed no signs of diarrhea or weight loss throughout the pretreatment period. During this period, animal weight increase was equal in all groups (data not shown). Pretreatment with probiotics attenuated deleterious effects of AP on nearly all parameters of liver injury. AP-induced increase in lipid peroxidation, AST and ALT was largely prevented after probiotic pretreatment (fig 2A, B, C). Dietary supplementation with probiotics ameliorated AP-induced liver injury[35] (fig 2E) attenuated necrosis (fig 2F), reduced apoptosis demonstrated by 36% reduction in DNA-fragmentation (fig 3A) and decreased activity of caspase-3 (fig 3B).

The found reduction in AP-induced activity of ICE and NF-κB (fig 4A, B) indicates that expression of pro-inflammatory factors was ameliorated after pretreatment with probiotics.

PROBIOTICS ENHANCED HEPATIC GLUTATHIONE CONTENT

As oxidative stress reflects the balance between ROS and anti-oxidative capacity, amelioration of oxidative stress-induced hepatic injury after probiotic pretreatment, as demonstrated by a reduction in lipid peroxidation (fig 2A), may have been due to either reduced amounts of ROS, or enhanced anti-oxidative defense systems. Therefore, oxidized glutathione (GSSG) and GSH were investigated. Firstly, in all pancreatitis groups GSSG increased two-fold compared to sham or control animals (fig 6A). Administration of probiotics did not prevent this increase, suggesting that both probiotic and placebo pretreated animals encountered a similar amount of ROS upon induction of AP. Secondly, pretreatment with probiotics prevented the AP-induced depletion of GSH content (fig 6B). Furthermore, the AP-induced decrease in GSH/GSSG ratio, indicating deleterious alterations in the cellular redox state, was partially prevented in probiotic pretreated animals (fig 6C). Of note, there was an inverse correlation between hepatic GSH content and histological scoring of liver injury ($r=-0.83$) (fig 6D), supporting the hypothesis that intracellular GSH protects against oxidative stress-induced liver injury. But most interestingly, probiotic pretreatment increased GSH levels, even compared with control and sham operated rats (fig 6B), suggesting an increase in anti-oxidative capacity after pretreatment with probiotics.

LOCAL BIOSYNTHESIS OF GLUTATHIONE

Intracellular GSH levels are mainly dependent on biosynthesis, which is regulated by availability of cysteine and GCL-activity[36]. Cysteine levels did not differ significantly between the groups (fig 7A). GCL-activity, however, was affected as AP per se increased GCL-activity compared to sham, but the most abundant increase was seen in probiotic pretreated animals which showed a 2-fold increase compared to controls (fig 7B), suggesting that GSH biosynthesis is enhanced by probiotics.

35 0 (0-1) vs. placebo 4 (4-6); $P<0.01$
36 Meister & Anderson, 1983

TABLE 1

Correlation coefficients between parameters of intestinal, pancreatic and hepatic injury

	Liver injury (Calabrese's score (1997))	**Hepatic inflammatory response (ICE activity)**	**Hepatic inflammatory response (NFκB activation)**
Intestinal barrier dysfunction			
Paracellular permeability (^{51}Cr-EDTA-flux)	0.90	0.73	0.81
Antigen passage (HRP-flux)	0.89	0.76	0.82
Bacterial passage (E. *coli* K12 flux)	0.94	0.85	0.83
Intestinal mucosal damage			
Mucosal damage (Chiu's score (1970))	0.85	0.75	0.64
Epithelial cell apoptosis (DNA-fragmentation)	0.94	0.72	0.71
Mucosal lipid peroxidation	0.96	0.84	0.78
Pancreatic pro-inflammatory release			
Pancreatic NFκB activation	0.68	0.72	0.39
Pancreatic ICE activity	0.61	0.75	0.48
Pancreatic injury			
Pancreatic damage (Spormann's score (1989))	0.67	0.70	0.34
Acinar cell apoptosis (DNA-fragmentation)	0.46	0.65	0.47
Pancreatic lipid peroxidation	0.60	0.70	0.39

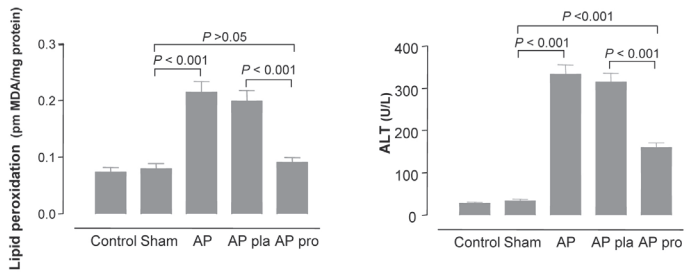
Correlations were computed using Spearman's rank correlation coefficients.

FIGURE 2

Probiotics reduced acute pancreatitis-induced hepatic injury

(A) Hepatic lipid peroxidation (MDA levels) and plasma levels of (B) ALT and (C) AST. The graphs show the average (±SEM). Comparisons were performed using ANOVA followed by Tukey's HSD. (D) Sections of liver samples, were H&E stained and graded according to Calabrese's scoring criteria (1997). Compared with sham operated animals, AP caused hepatocellular vacuolization, inflammatory infiltration and necrosis, which was reduced after probiotic pretreatment. The liver injury is typical of that seen in 4 sections from 12 rats per group. Bar=200μm. (E) Histopathological grading of hepatic injury. The graph shows the median (±range). Comparisons were performed using Kruskal-Wallis followed by Mann–Whitney *U* test. (F) The necrotic areas as percentages of the whole surface area.

A / B



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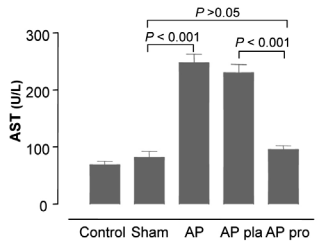

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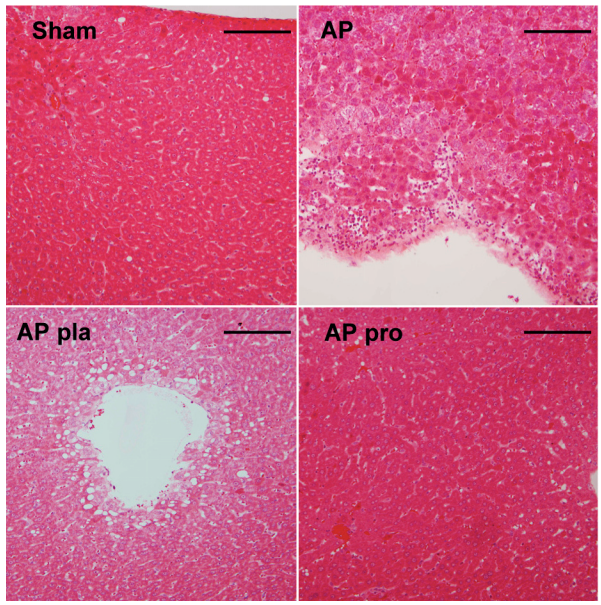

Sham
AP
AP pla
AP pro

E / F

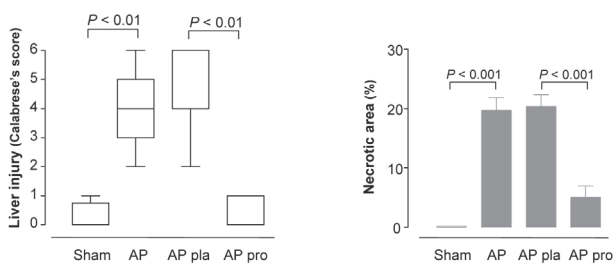

P < 0.01
P < 0.01
Liver injury (Calabrese's score)
0
1
2
3
4
5
6
Sham
AP
AP pla
AP pro
P < 0.001
P < 0.001
Necrotic area (%)
0
10
20
30
Sham
AP
AP pla
AP pro

FIGURE 3

Probiotics reduced acute pancreatitis-associated apoptosis in the liver

(A) Hepatic DNA fragmentation and (B) caspase 3 activation. The graphs show the average (±SEM). Comparisons were performed using ANOVA followed by Tukey's HSD.

A / B

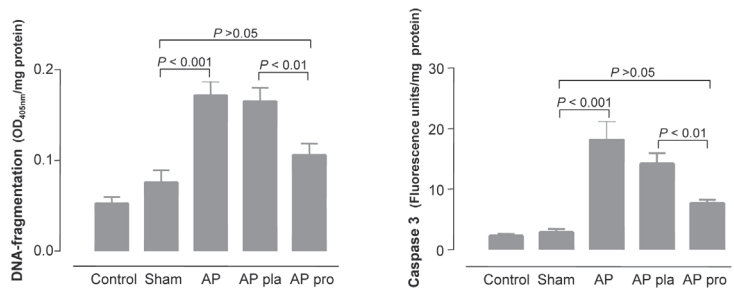


FIGURE 4

Probiotics reduced levels of acute pancreatitis-associated inflammatory markers

(A) Hepatic IL-1β-converting-enzyme (ICE) and (B) NF-κB-activation. The graphs show the average (±SEM). Comparisons were performed using ANOVA followed by Tukey's HSD. Correlations between lipid peroxidation and (C) ICE and (D) NF-κB-activation were computed using Spearman's rank correlation coefficients.

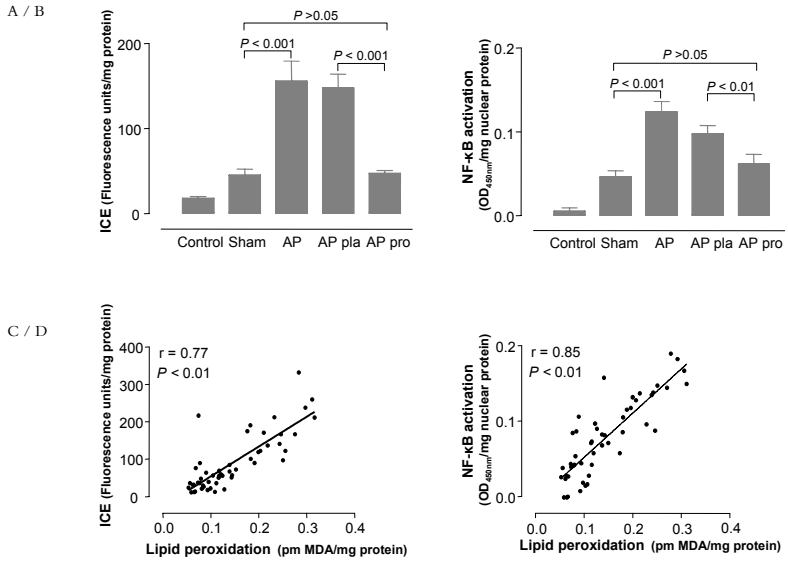


FIGURE 5

Correlations between acute pancreatitis-induced intestinal mucosal barrier dysfunction, pancreatic damage and liver injury

The three dimensional surface plot (B) shows associations between hepatic damage and pancreatic oxidative damage and hepatic damage and ileal oxidative damage. Correlation analysis between (A) liver injury, pancreatic lipid peroxidation and (C) ileal lipid peroxidation demonstrated that ileal oxidative damage (lipid peroxidation) correlates better with liver injury as compared to pancreatic lipid peroxidation. (B) This also showed in the 3D XYZ-plot, in which more extensive intestinal oxidative damage correlated with the severity of liver damage. In contrast to pancreatic lipid peroxidation; this did not seem to correlate very well with hepatic injury. Correlations were computed using Spearman's rank correlation coefficients.

A / B / C

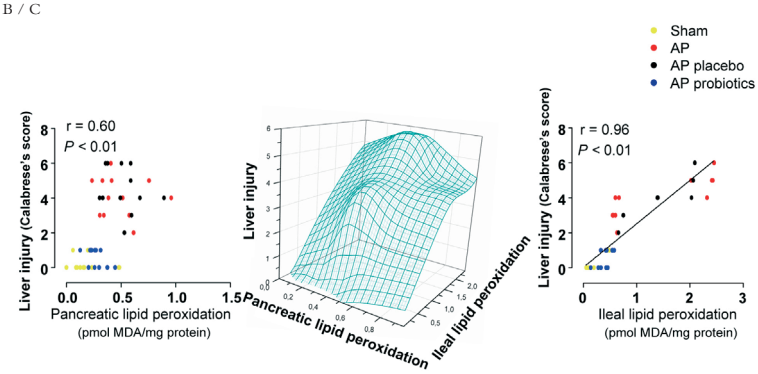


FIGURE 6

Probiotics reduced acute pancreatitis-induced oxidative stress

(A) Hepatic oxidized glutathione levels (GSSG) and (B) reduced glutathione levels (GSH). (C) GSH/GSSG ratios were calculated. The graphs show the average (±SEM). Comparisons were performed using ANOVA followed by Tukey's HSD. Correlations between (D) liver injury and GSH content were computed using Spearman's rank correlation coefficients.

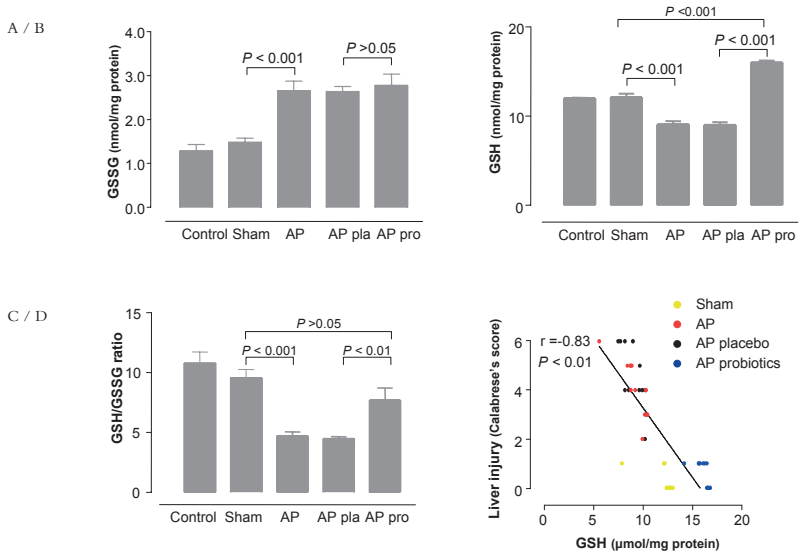
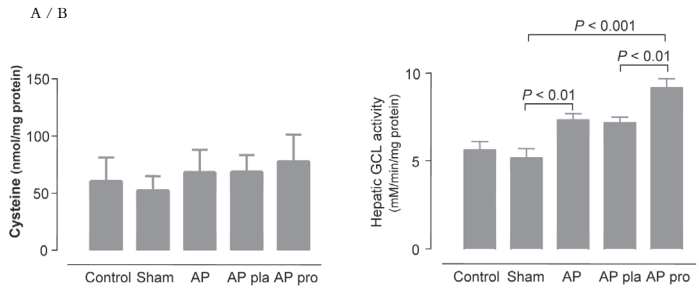


FIGURE 7

Probiotics have no effect on cysteine availability, but induce glutamate-cysteine-ligase activity

(A) Cysteine availability in the liver and (B) hepatic glutamate-cysteine-ligase (GCL) activity. The graphs show average (±SEM). Comparisons were performed using ANOVA followed by Tukey's HSD.



DISCUSSION

This study has demonstrated that administration of a selected mixture of probiotic bacteria stimulates hepatic GSH biosynthesis and enhances GSH levels, which resulted in amelioration of oxidative damage of the liver in the early phase of AP. In addition, GSH levels inversely correlated to hepatic injury scores, indicating that an increase in anti-oxidative capacity may be a potential mechanism behind the liver protective effects. This is still an unexplored mechanism of action of probiotics.

The present study is the first to demonstrate an increase in hepatic GSH content concurrent with local GSH biosynthesis after probiotic pretreatment. Oxidative stress plays a pivotal role in early AP. Within 5 minutes after onset of experimental pancreatitis, activation of neutrophils in the pancreas results in the generation of ROS[37], which spill over into the portal circulation and without any further dilution by the systemic circulation, cause extensive hepatic injury[38]. The major defense against oxidative stress is afforded by GSH, which levels depend on cysteine availability and GCL-activity. Cysteine is the rate-limiting precursor of GSH[39] and is found in most high protein foods. Cysteine availability was not affected by probiotic pretreatment. Therefore the enhanced activity of the rate-limiting enzyme GCL is the putative mechanism of elevated GSH levels found as a result of probiotic administration.

This finding however seems to be in contrast with results of the clinical trial conducted by our group, using the same combination of probiotic bacteria[40]. In this nation wide, randomized trial, administration of probiotics to severe AP patients resulted, unexpectedly, in increased intestinal ischemia and mortality. The critical difference between this randomized trial and our study is the timing of the administration of probiotics. Pretreatment was used as an experimental strategy in this study, in contrast to the clinical trial in which probiotics were administered after the onset of AP. Taking into account that enhanced GCL-activity can be expected in response to a sublethal oxidative stressor[41], administration of probiotics in these healthy animals may have been a small oxidative burden, consequently elevating GCL-activity and improving resistance toward subsequent potential lethal oxidative insults. In critically ill, AP patients however, the same small oxidative burden may have shifted their delicate redox balance for the worse. As oxidative stress levels have already reached critical heights in these severely ill patients, any extra (however small) oxidative burden may cause further oxidative damage instead of up-regulating antioxidative defense mechanisms. In support of this hypothesis, aggravation of intestinal mucosal barrier dysfunction due to probiotic treatment was found only in patients with multi-organ-failure[42].

In AP, ROS cause a decrease in tissue levels of hepatic GSH, succeed to substantially damage the liver and induce hepatocellular apoptosis[43]. And indeed, this study

37 Rau *et al.*, 2000
38 Folch *et al.*, 1998
39 Meister & Anderson, 1983
40 Besselink *et al.*, 2008
41 Lu, 2008
42 Besselink *et al.*, 2009
43 Folch-Puy, 2007

shows an inverse correlation between hepatic GSH levels and liver injury scores. Furthermore the liver is not only one of the affected organs in AP, but also forms a key contribution to the systemic inflammatory reaction[44]. In that regard, the liver is a unique organ because its resident Kupffer cells are the largest population of fixed tissue macrophages and it is seen as a predominant source of inflammatory cytokines in response to liver damage[45]. This is confirmed by the positive correlations between hepatic ICE and NFκB-levels and local lipid peroxidation found in this study.

Pro-inflammatory mediators and ROS derived from hepatocytes and Kuffer cells are in turn excreted from the liver into the systemic circulation and contribute significantly to multi-organ-failure. Gray *et al* (2003). showed that hepatic NF-κB is activated before AP-induced lung injury occurs. In that respect it is interesting that pretreatment with probiotics normalized hepatic ICE levels, considering that the liver releases about 50% of the total cytokine content[46].

Pancreatic elastase released during AP causes hepatic damage[47]. Other damaging substances may also be derived from the gastrointestinal tract. During AP, intestinal barrier dysfunction allows for endotoxins and bacteria to translocate into the portal circulation. Our group has previously shown that the used probiotics can limit bacterial translocation to extrapancreatic organs, including the liver in experimental pancreatitis[48] and prevent intestinal mucosal barrier dysfunction[49]. Correlation analysis showed that parameters of intestinal mucosal barrier dysfunction correlated better with liver injury scores and hepatic levels of pro-inflammatory mediators than factors of pancreatic damage suggesting that mucosal barrier dysfunction may be of greater importance to the amplification of the inflammatory signals through the liver.

In conclusion, our data show enhanced hepatic GSH biosynthesis after administration of probiotics. Our data further demonstrate that pretreatment with these multispecies probiotics, elevates hepatic GSH levels and consequently attenuates liver damage and activation of inflammatory mediators in a model of AP. Furthermore, the strong inverse correlation between hepatic GSH levels and liver injury scores found in this study further highlights the importance of this endogenous antioxidant defense system and sheds more light on this relatively new mechanism of action of probiotics. However, as probiotic properties are strain and species dependent, it must be stressed that the probiotic effects shown cannot automatically be extrapolated to others probiotic strains.

In addition, it should be stressed that oxidative damage plays a pivotal role very early in the course of AP. This vastly reduces the window of opportunity to employ probiotics to target oxidative stress and to prevent multi-organ-failure in the later course of the disease. Indeed, Siriwardena *et al.* (2007) demonstrated in a randomized trial that administration of anti-oxidative treatment to patients with predicted severe AP failed to show significant difference in organ dysfunction.

The effects of probiotics on GCL-activity found in this experiment most likely resulted from a mild oxidative stress. The same probiotic combination showed

44 Folch-Puy *et al.*, 2003
45 Gloor *et al.*, 2000
46 Shi *et al.*, 2006
47 Murr *et al.*, 2002
48 van Minnen *et al.*, 2007
49 Lutgendorff *et al.*, 2009

PROBIOTICS IN HEALTHY ANIMALS MAY HAVE BEEN A SMALL OXIDATIVE BURDEN, CONSEQUENTLY ELEVATING GCL-ACTIVITY AND IMPROVING RESISTANCE TOWARD SUBSEQUENT POTENTIAL LETHAL OXIDATIVE INSULTS

AS OXIDATIVE STRESS LEVELS HAVE ALREADY REACHED CRITICAL HEIGHTS IN SEVERELY ILL PATIENTS, ANY EXTRA OXIDATIVE BURDEN MAY CAUSE FURTHER DAMAGE

increased mortality in severe AP patients[50]. Therefore, this combination of probiotics is unfortunately not a treatment option in patients whose redox system is critically balanced such as critically ill patients. To benefit from the anti-oxidative properties of probiotics a preventive approach would be necessary. This could be applied in elective liver surgery in which intra-operative blood loss is typically prevented by inflow occlusion, inducing liver ischemia. The two current strategies to reduce ischemia-induced liver injury (i.e. ischemic preconditioning and intermittent clamping) both prolong resection time and increase operation risk. Therefore, non-invasive protective approaches are sought after particularly for livers with underlying diseases[51]. Indeed, pretreatment with probiotics has already shown positive results in a randomized trial in patients undergoing liver resection[52]. Based on results of this study, these positive clinical effects may have been caused by reduction of peroxidative liver injury, subsequent inflammatory response and upregulation of hepatic glutathione biosynthesis.

ACKNOWLEDGMENTS

Authors are most grateful to Martin B. de Smet for his skillful technical assistance. Winclove Bio Industries, Amsterdam supplied both the probiotics and placebo, but was however not involved in the design or in the publication of the study.

50 Besselink, 2008
51 Jang *et al.*, 2008
52 Sugawara *et al.*, 2006

8

Altered dendritic cell function and follicle-associated epithelial barrier in experimental acute pancreatitis restored by PPAR-γ-activating probiotics.

Probiotics modulate dendritic cells

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Harro M Timmerman [2] Hein G Gooszen [2]
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Submitted for publication

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ABSTRACT

BACKGROUND

In severe acute pancreatitis intestinal barrier dysfunction may be involved in secondary multiple organ dysfunction. Recently, dendritic cells in the subepithelial dome of the Peyer's patches were recognized as important players in mucosal immune responses. We hypothesized that experimental acute pancreatitis involves altered intestinal dendritic cell properties and mucosal barrier function in Peyer's patches and that probiotics prevent pancreatitis by activation of PPAR-γ[1], in dendritic cells.

METHODS

53 Spraque-Dawley rats were allocated into groups: 1) controls, non-operated, 2) sham-operated, 3) acute pancreatitis, 4) acute pancreatitis + probiotics (Ecologic® 641) and 5) acute pancreatitis + placebo. Acute pancreatitis was induced by intraductal glycodeoxycholate infusion and intravenous cerulein (6h). Daily probiotics or placebo were started five days prior to acute pancreatitis. *E. coli* K12 passage across ileal epithelium overlaying Peyer's patches was measured in Ussing chambers. PPAR-γ expression, dendritic cell-distribution and -maturation, and IL-12p40 expression were investigated by confocal immunofluorescence imaging. Ileal IL-12p40 levels and PPAR-γ activation were quantified.

1 peroxisome-proliferator-activated-receptor-γ

RESULTS

Acute pancreatitis induced increased bacterial passage[2], and *E. coli* invasion. Furthermore, acute pancreatitis caused a marked redistribution and maturation of dendritic cells[3], and increased IL-12 expression and mucosal IL-12p40 levels[4] in Peyer's patches. Probiotic pretreatment resulted in reduced IL-12p40 levels[5] and *E. coli* passage[6] concurrently with elevated levels of ileal PPAR-γ activation and enhanced PPAR-γ staining in dendritic cells.

CONCLUSIONS

Experimental acute pancreatitis induced dysfunction of the epithelial barrier and dendritic cells at the Peyer's patches. Probiotics enhanced ileal PPAR-γ activation, which putatively modulated dendritic cell distribution, decreased mucosal IL-12p40 levels and prevented the acute pancreatitis-induced intestinal barrier dysfunction.

2 sham 16.8 ± 2.2 *vs.* AP 96.9 ± 18.7 a.u.; $P<0.001$
3 CD103+CD86+
4 sham 12.4 ± 2.2 *vs.* AP 34.1 ± 4.6 pg/mg protein; $P<0.001$
5 probiotics 21.2 ± 4.1 *vs.* placebo 37.3 ± 6.1 pg/mg protein; $P<0.05$
6 probiotics 28.73 ± 6.7 *vs.* placebo 111.8 ± 24.4 a.u.; $P<0.001$

INTRODUCTION

Acute pancreatitis (AP) is a common condition of which the clinical presentation ranges from a mild edematous form to severe AP complicated with multi-organ-failure (MOF). In severe AP, the mucosal immune response plays a key role, not only as part of the systemic inflammatory response syndrome (SIRS)[7], but also as an aggravating factor in intestinal barrier dysfunction, giving the opportunity to endotoxins and bacteria to translocate into the systemic circulation, enhancing SIRS and closing the vicious circle[8]. Mucosal immune responses are mainly orchestrated by intestinal dendritic cells (DCs), which function to generate specific immune responses depending on their levels of co-stimulatory molecule expression and cytokine production profile[9]. Intestinal mucosal DCs mainly reside in the subepithelial dome of Peyer's patches, which is covered with specialized, M-cell containing follicle associated epithelium (FAE). FAE is, in contrast to regular villus epithelium more exposed to luminal contents[10], facilitating interaction between the lumen and the underlying DCs[11]. In addition, M-cells are specialized in controlled uptake of luminal contents and transportation of these antigens to the subepithelial dome where they are in close contact with the residing DCs[12]. Owing to its importance in initiating immune responses, the FAE with the underlying SED and its DCs have previously been studied in the context of inflammatory bowel diseases[13]. However the role of intestinal DCs in the early phase of AP has yet to be elucidated.

DCs are difficult to study *in vivo* because during isolation, maturation and other changes in gene expression that mimic an inflammatory state can be provoked[14]. However, immunohistochemical analysis enables researchers to study DCs in their physiological environment. Using immunohistochemical analysis an association between increased intestinal permeability and ileal DC redistribution has been demonstrated in acute and chronic experimental colitis[15]. Although mucosal barrier dysfunction has been shown to occur during acute pancreatitis[16], no data exist on redistribution of DCs under this condition.

Probiotics possess a whole array of mechanisms of action[17] and central to the mechanism of action of probiotics is their capacity to modulate the host immune responses. Although there is a considerable body of information concerning the immunomodulatory efficacy of probiotics, little is known about the precise mechanisms of action by which such bacteria may exert this property. Interactions with probiotics and DCs in the gut are believed to be able to differentially modulate DC maturation[18].

7 Frossard *et al.*, 2008
8 Clark & Coopersmith, 2007
9 Kalinski *et al.*, 2000; Pulendran *et al.*, 2001
10 Gebert, 1997
11 Neutra *et al.*, 2001
12 Keita *et al.*, 2006
13 Salim *et al.*, 2009; Keita *et al.*, 2008; Silva *et al.*, 2008; Spahn *et al.*, 2004
14 Liu & MacPherson, 1995
15 Silva *et al.*, 2006
16 Lutgendorff et al. 2009
17 Lutgendorff *et al.*, 2008
18 Christensen *et al.*, 2002; Hart *et al.*, 2004

THE MUCOSAL IMMUNE RESPONSE PLAYS A KEY ROLE IN SEVERE ACUTE PANCREATITIS

THE OBJECTIVE OF THE STUDY WAS TO PROVIDE THE FIRST EVIDENCE OF THE ROLE OF DENDRITIC CELLS IN ACUTE PANCREATITIS

Another suggested mechanism by which probiotics exert immunomodulatory properties, is the activation of peroxisome proliferator-activated receptor (PPAR)-γ[19], which has been shown to mediate anti-inflammatory effects[20]. Furthermore, there is a growing body of evidence that this anti-inflammatory effect of PPAR-γ is partially exerted via modulation of DC function, i.e. inhibition of maturation and down-regulation of IL-12 secretion[21].

The objective of the study was to investigate the AP-induced changes in FAE, regarding bacterial permeability, bacterial uptake into DCs, DC migration and alteration in cytokine profile, to provide the first evidence of the role of DCs in AP. Furthermore, we hypothesized that pretreatment with probiotics enhances PPAR-γ expression, modulates DC function and consequently ameliorates mucosal barrier dysfunction.

19 Ewaschuk *et al.*, 2006; Ewaschuk *et al.*, 2007
20 Ponferrada *et al.*, 2007
21 Klotz *et al.*, 2007; Faveeuw *et al.*, 2000

MATERIALS AND METHODS

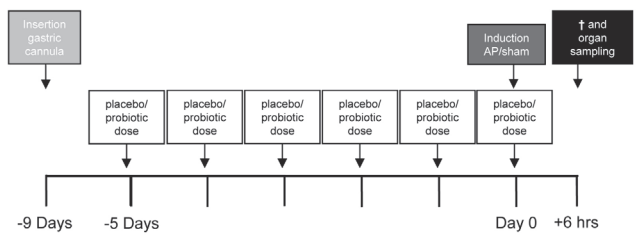
RATS

Male specific pathogen-free Sprague-Dawley rats (250-350 grams, B&K, Sollentuna, Sweden) were maintained under constant housing conditions (temperature (22°C), relative humidity (60%) and a 12-hour light/dark cycle) and had free access to water and standard rat chow throughout the experiment. The experimental design (fig. 1) was approved by the local committee of animal ethics. Fifty-three rats were randomly allocated into five groups: 1) non-operated control animals (n=5), 2) sham-procedure (n=12), 3) AP (n=12), 4) AP and placebo (n=12), 5) AP and probiotics (n=12).

FIGURE 1

Experimental design

Nine days prior to induction of pancreatitis, a gastric cannula was fitted into all animals, through which probiotics or placebo was administered intragastrically once daily, starting 5 days prior to induction of AP. After cerulein infusion, all animals were anesthetized for removal of organ samples.



SURGICAL PROCEDURES

At the start of the experiment, under general anesthesia using a combination of 2% Isoflurane gas (flow: 0.5 l/min O2, 1.5 l/min air), a permanent gastric cannula was fitted in all rats as described previously[22], except for non-operated control rats. Animals in the probiotics and placebo groups were allowed to recover for four days, prior to the start of daily probiotics or placebo administrations.

22 van Minnen *et al.*, 2007

The study product (*Ecologic® 641*, Winclove Bio Industries, Amsterdam, the Netherlands) consisted of six viable, freeze-dried strains: *Lactobacillus acidophilus* (W70), *Lactobacillus casei* (W56), *Lactobacillus salivarius* (W24), *Lactococcus lactis* (W58), *Bifidobacterium bifidum* (W23), and *Bifidobacterium lactis* (W52) and cornstarch and maltodextrin as carrier substances. Placebo, (the same substance without bacteria) was packed in identical sachets and coded by the producer to guarantee blinding. Directly before administration, products were reconstituted in sterile water, for 15 minutes at 37°C. Per probiotic dose $5x10^9$ CFU of bacteria were administered. Probiotics or placebo were daily administered through the gastric cannula, starting five days before induction of AP. The cannula was flushed with 1.0 ml water after administration of probiotics or placebo to ensure gastric delivery of the product. Animals in the sham and AP group were daily administered 1.0 ml water through the gastric cannula.

AP was induced as originally described by Schmidt *et al.* (1992) and previously performed by our group[23]. Pressure controlled retrograde infusion of 0.5 ml sterilized glycodeoxycholic acid (Sigma-Aldrich Chemie BV Zwijndrecht, The Netherlands) into the common bile duct was followed by intravenous infusion of cerulein (5µg/kg/h, 1ml/h, for 6 h, Sigma-Aldrich). No animals needed to be excluded for infusion pressures exceeding 35 mmHg. During the sham-procedure, the papilla of Vater was cannulated, but no glycodeoxycholic acid was infused, followed by 6 hours of intravenous saline infusion (1ml/hr).

COLLECTION AND PRESERVATION OF SAMPLES

After cerulein or saline infusion, rats were anaesthetized and 15 cm distal ileum and the pancreatic tail were harvested. Ten cm of ileum was allocated to Ussing chamber experiments and immediately submersed into ice-cold oxygenated Kreb's buffer (115mM NaCl, 1.25mM $CaCl_2$, 1.2mM $MgCl_2$, 2mM KH_2PO_4, and 25mM $NaHCO_3$, pH 7.35). Peyer's patches were identified and samples for histological and immunohistochemical examination were formalin (4%) fixed over night, and stored in 30% sucrose before embedding in optimum cutting temperature compound (Histolab, Västra Frölunda, Sweden). A small portion of the pancreatic tail was harvested and fixed in 4% formalin. Mesenteric lymph nodes (MLNs) and mucosal scrapings from ileal epithelium overlaying Peyer's patches were snap frozen and stored at -80°C. All analyses were run in duplicates.

HISTOLOGY

To confirm AP, pancreatic tail sections (2µm) were hematoxylin-eosin (H&E) stained and scored according to Spormann's criteria for pancreatic injury[24].

23 Lutgendorff *et al.*, 2009
24 Spormann *et al.*, 1989

USSING CHAMBER EXPERIMENTS

Permeability of follicle associated epithelium (FAE) was measured as previously described[25]. Briefly, FAE was identified, stripped of external muscle while immersed in Kreb's buffer, and mounted into Ussing chambers (Harvard Apparatus Inc., Holliston, MA, USA)[26]. A tissue area of 9.6mm^2 exposed to 3ml circulating, oxygenated Kreb's solution at 37°C. To meet physiological conditions, the serosal buffer contained 10mM glucose which was osmotically balanced by 10mM mannitol in the mucosal buffer. Vitality was assessed by monitoring potential difference across the tissue using agar-salt bridges. Forty minutes after mounting steady-state conditions were reached and base-line values for short circuit current (Isc), indicating net ion secretion, and conductance (passive ion flux), were recorded.

Horseradish peroxidase (HRP) (Sigma-Aldrich) was used as model antigen to determine transepithelial transport of macromolecules, and ^{51}Cr-EDTA (Perkin-Elmer, Boston, MA, USA) fluxes were measured to assed paracellular permeability. HRP and ^{51}Cr-EDTA were added to the mucosal side to a final concentration of 10^{-5} M and 34μC$_i$/ml, respectively. Serosal samples (300μl) were collected at 0, 30, 60, 90 and 120 min after start and were used to analyze transepithelial fluxes of ^{51}Cr-EDTA, expressed as cm/s·10^{-6}, using a gamma-counter (1282 Compugamma, LKB, Bromma, Sweden). HRP-activity was determined as previously described[27], and transepithelial HRP flux was expressed as pmol/cm^2/h. Permeability was calculated in 3 ileal samples per rat during the 30–120 min period for both markers.

To assess bacterial passage, fluorescent *E. coli* K12 (1x10^8CFU/ml, Molecular Probes, Leiden, the Netherlands), killed by paraformaldehyde to stop reproduction without loss of antigenicity[28] were added after equilibration to the mucosal side. After 120 min, the entire volume of serosal compartments was analyzed at 488 nm in a fluorimeter (Cary Eclipse, Varian, Victoria, Australia). One unit corresponds to 3.0·10^3 CFU/ml[29].

MUCOSAL INVASION AND ADHESION OF *E. COLI* K12

To study invasion and mucosal adhesion of *E. coli* K12, FAE was mounted into Ussing chambers following the procedure described above and was exposed to 1x10^8 CFU/ml fluorescent *E. coli* K12 for 10 min. Tissues were subsequently washed with Krebs buffer, dismounted, placed in OCT, and stored at -80°C until being processed for immunohistochemistry.

IMMUNOHISTOCHEMISTRY DOUBLE STAINING

Immunohistochemistry double staining was performed on frozen sections (5μm) of ileal Peyer's Patches as previously published[30]. Briefly, 4 sections of 4 rats per group were rehydrated with PBS and subsequently treated with 10% bovine serum albumin, washed and over night incubated at 4°C either with 1:50 mouse anti-rat

25 Lutgendorff *et al.*, 2009
26 Grass & Sweetana, 1988
27 Velin *et al.*, 2004
28 Velin *et al.*, 2004
29 Velin *et al.*, 2004
30 Salim *et al.*, 2009

CD103 or with one of the following pairs of antibodies: 1) 1:50 mouse anti-rat CD103 + 1:50 rabbit anti-rat CD86 2) 1:50 mouse anti-rat CD103 + 1:50 goat anti-rat IL-12p40 3) 1:50 mouse anti-rat CD103 + 1:50 rabbit anti-rat PPAR-γ. Mouse anti-rat CD103 and goat anti-rat IL-12p40 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA and rabbit anti-rat CD86 and rabbit anti-rat PPAR-γ were purchased from Abcam Inc., Cambridge, USA. Following extensive washes, slides were incubated with Alexa Fluor®633 goat anti mouse IgG and Alexa Fluor®488 donkey anti-rabbit or Alexa Fluor®488 donkey anti-goat (1:500 dilution, Jackson ImmunoResearch Europe Ltd, London, United Kingdom) for 1hr at room temperature.

All sections were washed and certain sections were counterstained with Alexa Fluor®581-conjugated phalloidin for 10min, mounted in anti-fading Fluorescent Mounting Medium (DakoCytomation, Stockholm, Sweden) and examined using confocal imaging with a 2-photon BioRad Radiance 2000 microscope (Carl Zeiss, Jena, Germany), equipped with high numerical aperture (NA=1.4) 60× and 100× oil immersion objectives. Each test included negative controls. Image acquisition settings were identical for each experiment.

DCs were quantified by counting the number of CD103 positive cells within a defined area that covered the subepithelial dome (SED) using Nikon EZ-C1 software. Results are presented as the number of DCs per mm^2. Maturation rate is expressed by the number of CD103/CD86 positive cells per total of CD103 positive cells. Expression of IL-12 in DCs was quantified by counting the number of IL-12 positive DCs and expressed as percentage total number of DCs per area unit. Invading and adherent *E. coli* were counted within a subepithelial predefined area and expressed as number of bacteria/mm^2. All quantifications were done in 4 randomly selected sections from 4 rats per group.

IL-10 AND IL-12p40 LEVELS

IL-10 and IL-12p40 levels were determined in mucosal scrapings and MLNs utilizing commercially available ELISA kits (Invitrogen, Carlsbad, CA, USA). Briefly, tissue was homogenized and after centrifugation (10,000x*g,* 10min, 4°C) protein contents were measured using Bradford's method[31]. IL-10 and IL-12p40 levels were determined in the cytosolic protein fraction, according to manufacturer's instructions. Results are expressed as pg/mg tissue.

PPAR-γ ACTIVATION

To determine activated PPAR-γ, PPAR-γ transcription factor levels were determined in the nuclear fraction of mucosal scrapings. Nuclear extraction was performed as previously described by our group[32] and PPAR-γ transcription factor levels were quantified using a rat PPAR-γ transcription factor ELISA kit (USCN LIFE, Missouri, TX, USA) as indicated by the manufacturer. Results were normalized to nuclear protein and are expressed as ratio to controls.

31 Bradford, 1976
32 Trulsson *et al.*, 2004

STATISTICAL ANALYSIS

All data were assessed for normal distribution using Shapiro–Wilk's test. Parametric values are presented as mean (SEM). Statistical analysis was performed by ANOVA followed by Tukey's HSD test for multiple comparisons (SPSS 13.0 Inc, Chicago, Illinois, USA). Non-parametric values are given as median (25-75th interquartile range). Comparisons were done by Kruskal-Wallis when more than two groups were compared. Mann-Whitney U test was used to compare two groups. Spearman's rank correlation coefficients were computed for correlation analyses. *P* values <0.01 were considered significant.

RESULTS

ACUTE PANCREATITIS CAUSED DENDRITIC CELL MATURATION, MIGRATION AND CYTOKINE EXPRESSION.

Induction of AP did not cause any mortality and was confirmed by histological scoring of pancreatic damage[33].

Induction of AP resulted in marked increase in bacterial adherence to FAE (red arrowheads, fig 2A, B), which is the first step in invasion and penetration of bacteria through the mucosal surface[34]. And indeed, a 5 fold increase in bacterial passage of *E. coli* K12 through the FAE was demonstrated in AP subjected animals (fig 2C). Bacterial uptake was confirmed by confocal microscopy which revealed bacteria invading the epithelium (white arrowheads fig 2A, D). In addition, after induction of AP, permeability to HRP in the FAE increased by as much as 4 fold, which was accompanied by an increase in paracellular ^{51}Cr-EDTA flux, elevated short circuit current, and increased baseline conductance, representing paracellular ion flux (table 1).

It has been shown in experimental colitis that upon the enhanced antigen and bacterial uptake which is a direct consequence of increased permeability of FAE, DCs mature and migrate from the SED to the mesenteric lymph nodes (MLN) rendering the intestine even more susceptible to immune activation and inflammation by a deficient regulatory and/or an excessive effector cell response[35]. To elucidate this phenomenon in AP, DCs were quantified in sections stained for CD103, a specific DC marker. Laser confocal microscopy imaging revealed a vast decrease in the number of DCs in the SED after induction of pancreatitis (fig 3 A, B). In contrast, double staining with CD103 and CD86 which is expressed on mature DCs showed that maturation, expressed as percentage CD86/CD103 positive cells out of the total of CD103 positive cells was increased in animals subjected to AP (fig 3 C, D). Of note, in all animals DCs internalized fluorescent *E. coli* K12, however this was more frequent in animals subjected to AP (fig 4).

During their maturation DCs are able to modulate the T-cell mediated immune response through differential expression and production of IL-12p40 and IL-10[36]. Induction of AP caused an increase in IL-12p40 in FAE (fig 5A). Interestingly, double staining for CD103 and IL-12 demonstrated IL-12 positive DCs after induction of AP which were absent in sham animals (fig 5B, C). In mucosal scrapings from FAE, IL-10 levels were also increased after induction of AP (fig 5D).

Upon antigen captivation and subsequent maturation, DCs migrate to MLNs where they activate and differentiate T-cells based on their cytokine production. It was therefore of interest to determine cytokine levels in MLN tissue. In AP subjected animals, IL-12 levels showed a 10 fold increase and also IL-10 levels showed a marked increase after induction of AP (fig 5E, F).

33 0 (0-0) *vs.* 3 (2-5.1) after induction of pancreatitis; $P<0.001$
34 Lu & Walker, 2001
35 Silva *et al.*, 2006
36 Kalinski *et al.*, 2000

PROBIOTICS MODULATED DENDRITIC CELL MATURATION AND CYTOKINE EXPRESSION

No rats receiving probiotics showed signs of diarrhea, weight loss or loss of appetite during the pretreatment period. Increase in animal weight was similar in all groups[37].

Five days of probiotic administration resulted in attenuation of increased permeability of FAE induced by AP (table 2). Furthermore, the AP-induced breach in mucosal barrier to bacteria was decreased in the probiotic treated animals (fig 6A), which was also revealed by laser confocal imaging showing a decrease in bacterial invasion into the follicle associated epithelial layer (fig 6B, table 3). Also, AP-induced bacterial adhesion was decreased after probiotic pretreatment (fig 6B, table 3) however pretreatment with probiotics did not have an effect on the increase in Isc induced by AP (table 2).

Probiotic pretreatment prevented the decrease in the number of DCs in the SED after induction of pancreatitis (fig 6C, table 3) and compared with placebo treated animals, probiotic pretreatment reduced the number of maturated DCs (fig 6D, table 3). In addition, enhanced internalization of *E. coli* K12 by DCs was reduced in the probiotic treated group (fig 6E, table 3).

Depending on the cytokine profile that is expressed by DCs upon maturation the subsequent T-cell response is polarized. IL-10 is implicated in priming Th2 responses, while IL-12 potently induces gamma interferon (IFN-γ)-producing Th1 cells[38]. Pretreatment with probiotics decreased the AP-induced elevation of IL-12 both locally and in MLNs (fig7 A, E), concordantly with the laser confocal micrographs which showed a decrease in IL-12 positive DCs in the SED (fig 7 B, C). In contrast, probiotic pretreatment only further enhanced the elevation in local and systemic IL-10 levels induced by AP (fig 7 D, F) indicating that probiotics modulated DC cytokine production.

PROBIOTICS ENHANCED EPITHELIAL PPAR-γ LEVELS

As PPAR-γ activation is involved in DC modulation[39] and maintenance of mucosal barrier integrity, it was of interest to determine the effects of probiotic pretreatment on mucosal PPAR-γ. Pretreatment with probiotics increased mucosal PPAR-γ transcription factor almost 2 fold (fig 8A). As shown by immunohistochemical double staining of CD103 and PPAR-γ, elevation in PPAR-γ levels after probiotic treatment was not only located in the epithelial cell layer, but also within DCs in the SED (fig 8B). AP, however did not have an effect on mucosal PPAR-γ levels (fig 8 A, B).

Of note, in AP subjected animals there was positive correlation between mucosal PPAR-γ levels and mucosal IL-10 levels (r=0.73) (fig 8C) and an inverse association between PPAR-γ levels and mucosal IL-12 levels (r=-0.83), supporting the hypothesis that PPAR-γ activation exerts immunomodulatory properties.

37 sham-operated 22.3 (1.09) *vs.* AP 21.9 (1.11) *vs.* placebo 20.6 (0.82) *vs.* probiotics 20.5 (0.61)
38 Kalinski *et al.*, 2000
39 Klotz *et al.*, 2007; Faveeuw *et al.*, 2000

TABLE 1

Acute pancreatitis caused increased permeability of follicle associated epithelium

	HRP flux (pmol/cm^2/h)	**^{51}Cr-EDTA flux** (cm/s(x10^{-6}))	**Conductance** (mS/cm^2)	**Current** (μA/cm^2)
Sham (n=12)	33.9 (5.1)	6.8 (1.2)	12.4 (0.8)	23.5 (4.6)
Acute pancreatitis (n=12)	133.8 (11.6)*	19.1 (2.5)*	21.0 (1.9)*	42.4 (6.1)*

Rats were subjected to acute pancreatitis or underwent a sham procedure. Barrier integrity of follicle associated epithelium was tested in Ussing chambers. Values are shown mean (SEM). *P< 0.01 compared to sham animals.

TABLE 2

Pretreatment with probiotics prevent acute pancreatitis-induced barrier dysfunction in FAE

	HRP flux (pmol/cm^2/h)	**51Cr-EDTA flux** (cm/s(x10^{-6}))	**Conductance** (mS/cm^2)	**Current** (μA/cm^2)
AP, placebo (n=12)	128.2 (11.3)	16.1 (2.1)	20.8 (1.8)	44.5 (7.4)
AP, probiotics (n=12)	62.2 (16.2)*	6.7(0.4)*	14.7 (0.6)*	42.5 (8.3)*

AP, acute pancreatitis; FAE, follicle associated epithelium; Rats were pretreated with probiotics or placebo and subjected to acute pancreatitis. Barrier integrity of follicle associated epithelium was tested in Ussing chambers. Values are shown mean (SEM). *P< 0.01 compared to placebo treated animals.

TABLE 3

Pretreatment with probiotics modulates dendritic cell function

	Invading bacteria (No./mm^2)	**Adherent bacteria** (No./mm^2)	**Dendritic cells** (No./mm^2)	**DCs internalizing *E. coli*** (%)	**CD86+ DCs** (%)
AP, placebo (n=12)	56 (9 -121)	107 (19-146)	19 (9-47)	10.4 (7.1-16.7)	40.4 (22.2-50.3)
AP, probiotics (n=12)	0 (0 -16)*	42 (20-64)*	72 (37-215)*	2.6 (1.0-4.1)*	6.1 (0.0-9.4)*

AP, acute pancreatitis; FAE, follicle associated epithelium; Rats were pretreated with probiotics or placebo and subjected to acute pancreatitis and adhesion and invasion as well as DC number and function were observed by confocal microscopy. Values are shown median (range). *P< 0.01 compared to sham animals.

FIGURE 2

Acute pancreatitis caused increased bacterial adhesion and invasion

Rats were subjected to acute pancreatitis (AP, n=12), a sham-procedure (n=12) or not operated (control, n=5). Follicle-associated epithelium, covering Peyer's patches was mounted in Ussing chambers and exposed to fluorescent *Escherichia coli* K12 (green) for 10 min. Actin was visualized by fluorescently labeled phalloidin (red). (A) AP induced invasion of *E. coli* into the subepithelial layer (white arrowheads) and bacterial adhesion to follicle-associated epithelium (red arrowheads). Bar=200µm. The patterns of staining are typical of that seen in 4 sections of 4 rats per group. (B) Quantification of *E. coli*, adherent to the mucosa and (C) invading the mucosa. (D) Passage of *E. coli* K12 through follicle associated epithelium was studied for two hours in Ussing chambers. The graphs show average (±SEM). The data were collected from independently acquired sets of 3 tissue segments per rat. Comparisons were performed using ANOVA followed by Tukey's HSD. (C) Quantification of bacterial adherence.

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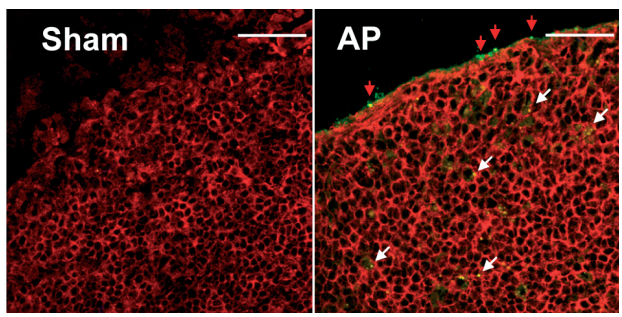

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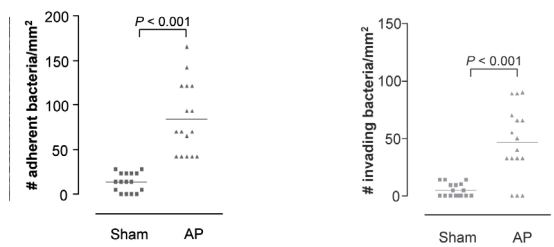

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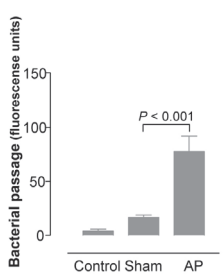

FIGURE 3

Acute pancreatitis induces migration of dendritic cells away from the subepithelial dome

Rats were subjected to acute pancreatitis (AP), or a sham-procedure. Ileal epithelium, covering Peyer's patches was processed for confocal microscopy. (A) Sections were stained with CD103 (dendritic cells, blue) and counterstained with fluorescently labeled phalloidin (actin, red). Bar=200μm. AP caused a marked decrease in the number of dendritic cells in the subepithelial dome. (B) Sections were double stained for CD103 (dendritic cells, blue) and the co-stimulatory molecule CD86 (green). CD86 was mainly localized in the cytoplasm of dendritic cells, but was however more abundant after induction of AP (arrowheads). Bar=100μm. (C) Quantification of dendritic cells in the subepithelial dome. (D) Quantification of CD86 positive dendritic cells expressed as percentage of total numbers of dendritic cells. The patterns of staining are typical of that seen in 4 sections of 4 rats per group.

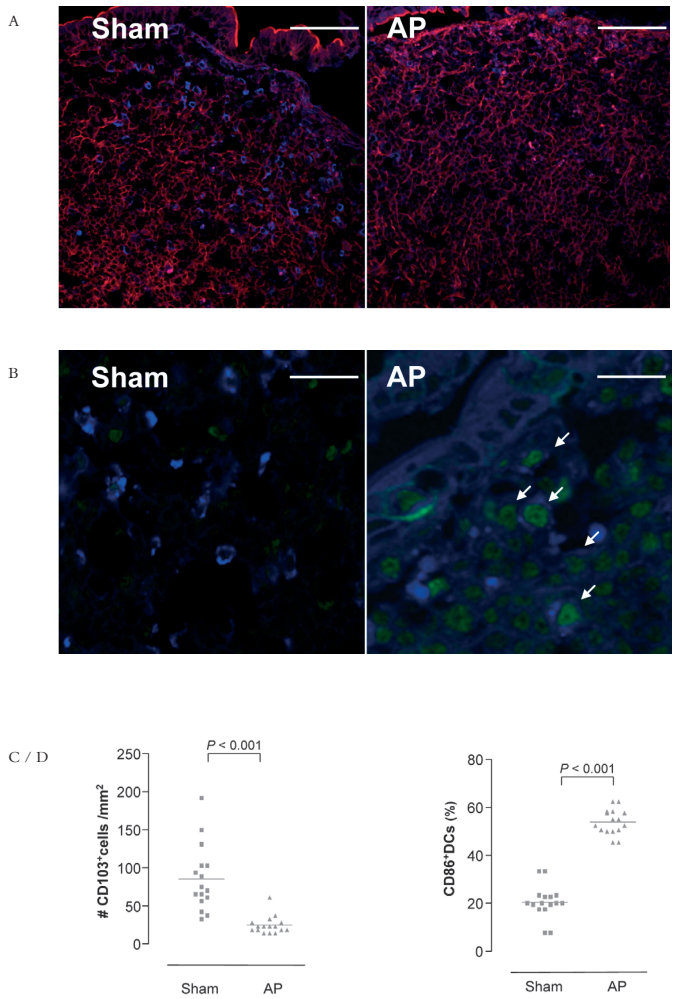

FIGURE 4

Acute pancreatitis induced enhanced internalization of *E. coli* K12 by dendritic cells

Rats were subjected to acute pancreatitis (AP), or a sham-procedure. Ileal epithelium, covering Peyer's patches was mounted in Ussing chambers and exposed to fluorescent *E. coli* K12 (green) for 10 min before being processed for confocal microscopy and stained for CD103 (dendritic cells, blue). (A) In animals subjected to AP, dendritic cells were found to internalize *E. coli* more frequently as compared to the sham group. Bar=150µm. Insets are magnifications of areas indicated by arrowheads. (B) Quantification of the frequency of internalization of *E. coli* by dendritic cells, expressed as percent of dendritic cells that co-localized with *E. coli*. Quantifications are based on random micrographs of 4 sections of 4 rats per group.

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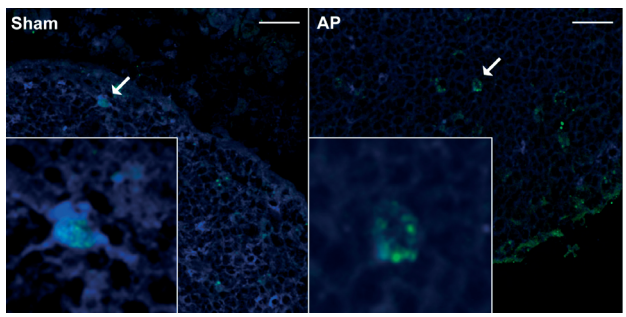

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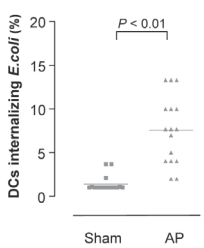

FIGURE 5

Acute pancreatitis induces a Th1 type response

Rats were subjected to acute pancreatitis (AP, n=12), a sham-procedure (n=12) or not operated (control, n=5). Peyer's patches were identified excised and mucosal scrapings were processed for biochemical analysis. (A) IL-12 levels. (B) Sections of follicle-associated epithelium were double stained for CD103 (dendritic cells, blue) and IL-12 (green) and counterstained with phalloidin (actin, red). AP increased the number of IL-12 expressing dendritic cells (pink, arrowheads). Bar=200μm. The patterns of staining are typical of that seen in 4 sections of 4 rats per group (C) Quantification of IL-12 positive dendritic cells, expressed as percentage of total number of dendritic cells. (D) IL-10 levels in mucosal scrapings from Peyer's patches. In addition, mesenteric lymph nodes were harvested and (E) IL-12 and (F) IL-10 levels were determined. The graphs show the average (±SEM). All analyses were run in duplicates. Comparisons were performed using ANOVA followed by Tukey's HSD.

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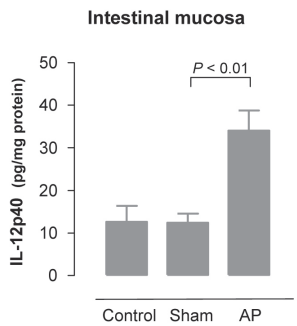

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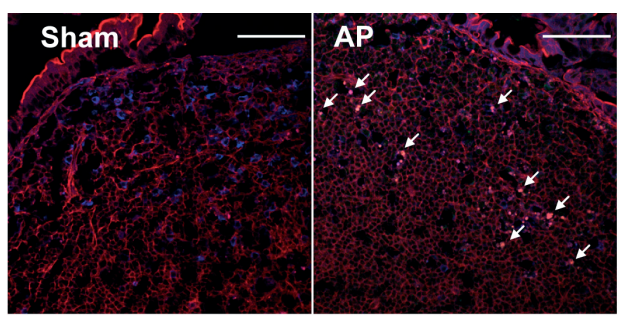
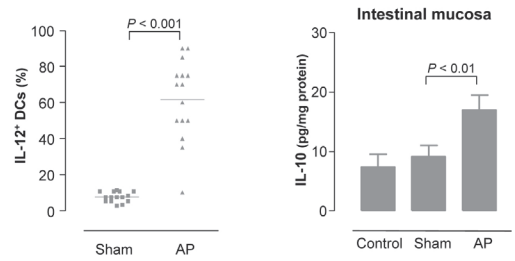


FIGURE 5

C / D



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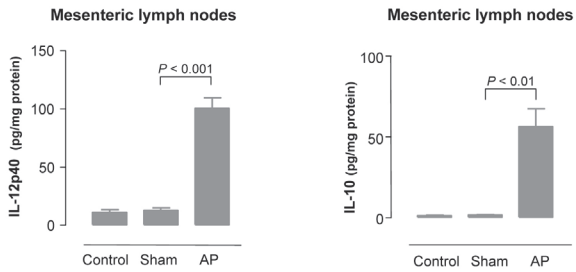

FIGURE 6

Probiotics modulate dendritic cell function

After 5 days of pretreatment with placebo (pla, n=12) or probiotics (pro, n=12), rats were subjected to acute pancreatitis (AP). Ileal epithelium, covering Peyer's patches was mounted in Ussing chambers and (A) passage of *Escherichia coli* K12 was studied for two hours. The graphs show average (±SEM). The data were collected from independently acquired sets of 3 tissue segments per rat. Comparisons were performed using ANOVA followed by Tukey's HSD. (B) Separate tissue segments were exposed to fluorescent *E. coli* K12 (green) for 10 min and actin was visualized by fluorescently labeled phalloidin (red). Probiotics prevented the AP-induced invasion of *E. coli* into the subepithelial layer (white arrowheads) and reduced bacterial adhesion (red arrowheads). Bar=200μm (C) An additional set of follicle-associated epithelial sections were stained with CD103 (dendritic cells, blue) and counterstained with fluorescently labeled phalloidin (actin, red). The AP-induced decrease in dendritic cells in the subepithelial dome was normalized by probiotic treatment. Bar=200μm. (D) Sections were double stained for CD103 (dendritic cells, blue) and the co-stimulatory molecule CD86 (green). Pretreatment with probiotics reduced the abundance of CD86 positive dendritic cells (arrowheads). Bar=100μm. (E) Tissue exposed to fluorescent *E. coli* K12 (green) was stained for CD103 (dendritic cells, blue). Probiotics reduced the AP-induced enhanced internalization of bacteria. Bar=150μm. Insets are magnifications of areas indicated by arrowheads. The patterns of staining are typical of that seen in 4 sections of 4 rats per group.

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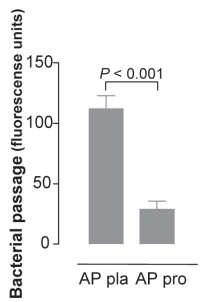

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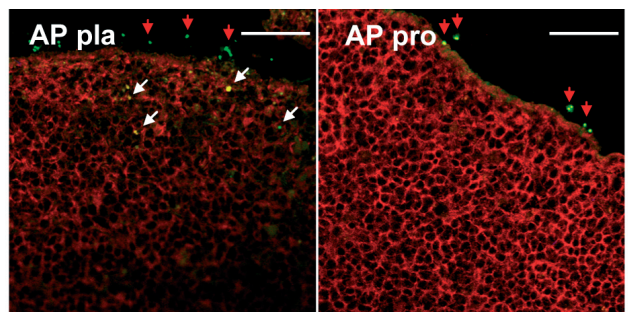

FIGURE 6

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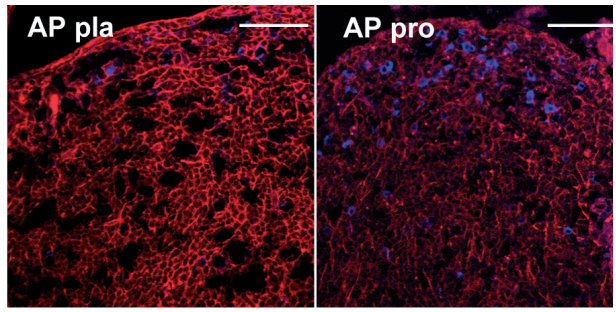

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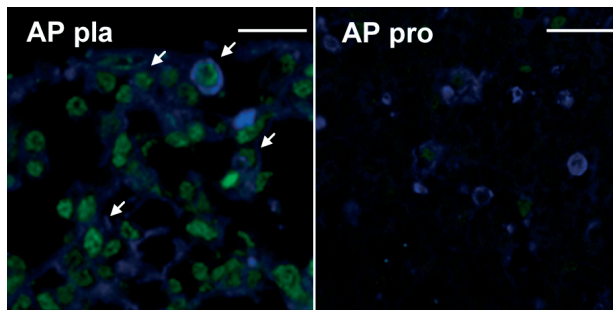

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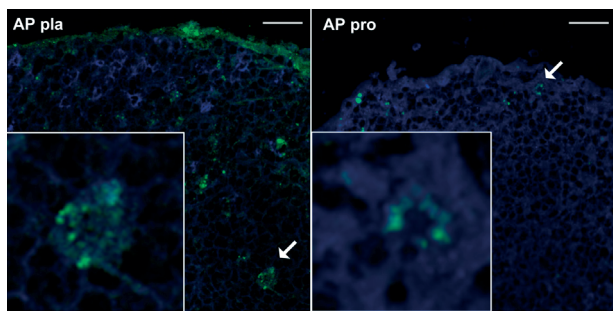

FIGURE 7

Effects of probiotics on Il-12 and IL-10 levels

After 5 days of pretreatment with placebo (pla, n=12) or probiotics (pro, n=12), rats were subjected to acute pancreatitis (AP, n=12), a sham-procedure (n=12) or not operated (control, n=5). Peyer's patches were identified excised and mucosal scrapings were processed for biochemical analysis. (A) IL-12 levels. (B) Sections of follicle-associated epithelium were double stained for CD103 (dendritic cells, blue) and IL-12 (green) and counterstained with phalloidin (actin, red). Probiotics prevented the AP-induced increase in the number of IL-12 expressing dendritic cells (pink, arrowheads). Bar=200µm. The patterns of staining are typical of that seen in 4 sections of 4 rats per group (C) Quantification of IL-12 positive dendritic cells, expressed as percentage of total number of dendritic cells. (D) IL-10 levels in mucosal scrapings from Peyer's patches. Also mesenteric lymph nodes were harvested and (E) IL-12 and (F) IL-10 levels were determined. The graphs show the average (±SEM). All analyses were run in duplicates. Comparisons were performed using ANOVA followed by Tukey's HSD.

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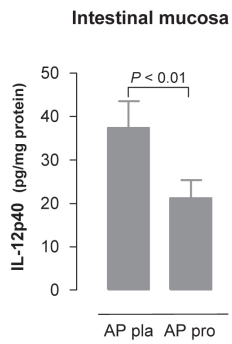

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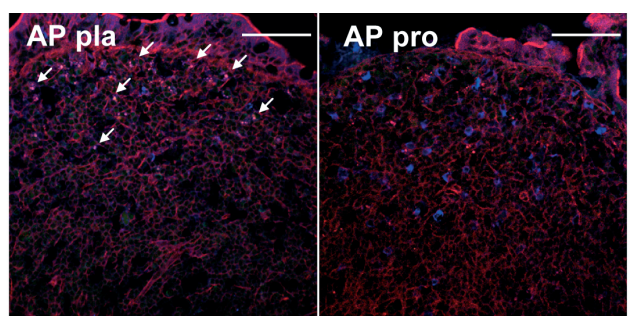

FIGURE 7

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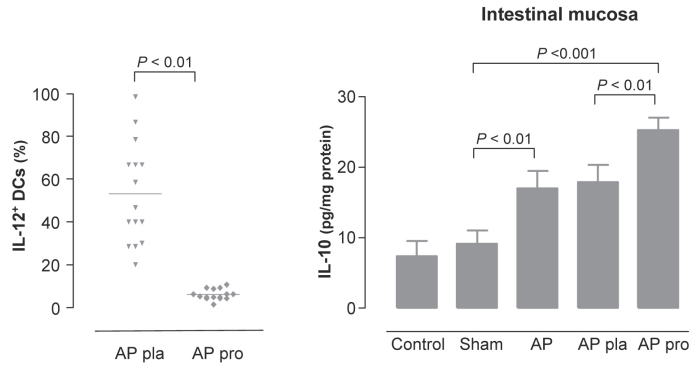

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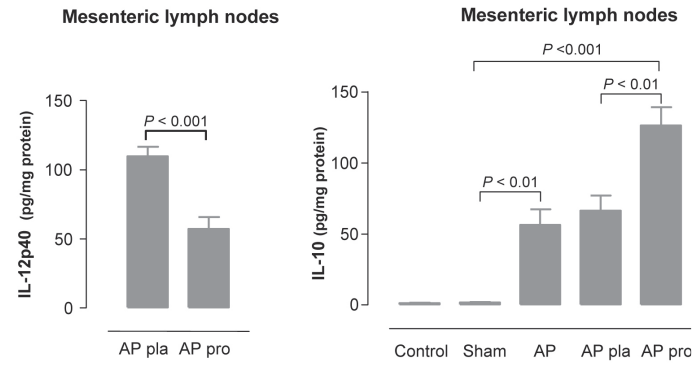

FIGURE 8

Probiotics enhanced PPAR-γ expression in dendritic cells.

After 5 days of pretreatment with placebo (pla, n=12) or probiotics (pro, n=12), rats were subjected to acute pancreatitis (AP, n=12), a sham-procedure (n=12) or not operated (control, n=5). Six hours after induction of the AP or sham-procedure Peyer's patches were identified excised and mucosal scrapings were processed for biochemical analysis. (A) PPAR-γ transcription factor levels in mucosal scrapings. The graph shows the average (±SEM). All analyses were run in duplicates. Comparisons were performed using ANOVA followed by Tukey's HSD. (B) Sections of follicle-associated epithelium were double stained for CD103 (dendritic cells, blue) and PPAR-γ (green). AP did not have any effects on staining intensity of PPAR-γ. Probiotic administration, however, mildly increased PPAR-γ levels in the cytoplasm of epithelial cells and induced a vast increase in the number of PPAR-γ positive dendritic cells (turquoise) in the subepithelial dome. Bar=200μm. The patterns of staining are typical of that seen in 4 sections of 4 rats per group. Correlations between mucosal levels of PPAR-γ transcription factor and (C) mucosal IL-10 and (D) mucosal IL-12 were computed using Spearman's rank correlation coefficients.

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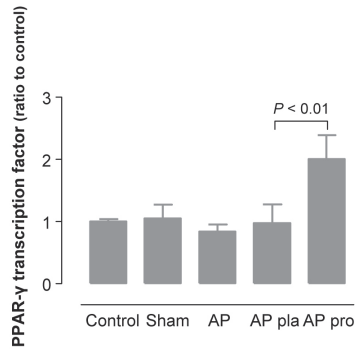

FIGURE 8

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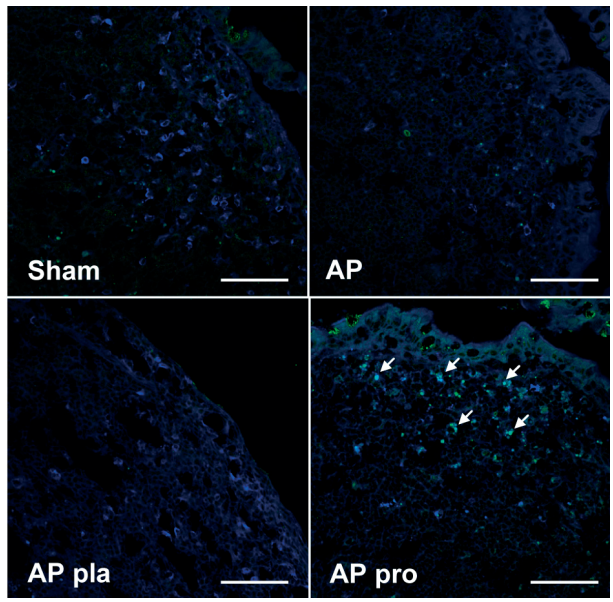
Sham
AP
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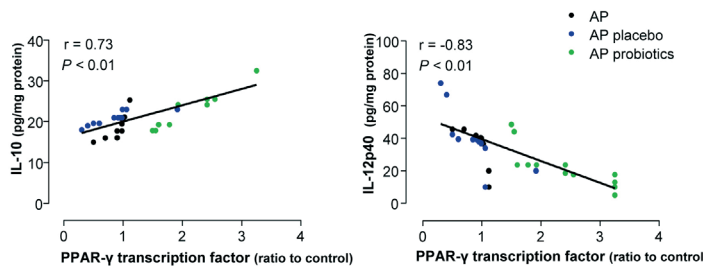
r = 0.73
P < 0.01
IL-10 (pg/mg protein)
PPAR-γ transcription factor (ratio to control)
r = -0.83
P < 0.01
IL-12p40 (pg/mg protein)
PPAR-γ transcription factor (ratio to control)
AP
AP placebo
AP probiotics

DISCUSSION

Intestinal mucosal DCs play a pivotal part in the initiation and orchestration of mucosal immune responses. Although knowledge is accumulating rapidly on their role in intestinal inflammation, little is known about their role in AP in which intestinal mucosal immune responses are the determining factors in disease severity[40]. This study is the first to provide evidence that upon AP-induced bacterial adhesion, DCs internalize bacteria, mature and express a Th1 type cytokine profile. Additionally, we observed that pretreatment with probiotics enhances mucosal PPAR-γ activation, modulates DC function and immune response and ameliorates AP-induced barrier dysfunction of epithelium overlaying Peyer's patches in rats.

The intestinal immune system forms the largest part of the immune system in which DCs are mainly responsible for bacterial and antigen detection and presentation and have the unique capability to stimulate naive T-cells and to shape their immune response[41]. From animal studies it is known that this mainly takes place in specialized lymph follicles and is facilitated by FAE[42]. FAE differs form the surrounding epithelium. It lacks goblet cells, IgA transport ability and has low levels of hydrolases, creating an environment more conductive to uptake of various microorganisms[43]. Under steady-state conditions, intestinal DCs interact directly with luminal bacteria by passing their dendrites past epithelial tight junctions into the gut lumen[44] and indirectly interact with bacteria that have gained access through M-cells specialized in uptake of bacteria and antigens. In this study we have provided novel *ex vivo* evidence that AP induces bacterial adherence to FAE, which is the first and essential step in the onset of the inflammatory reaction, since it facilitates penetration and invasion of bacteria into the subepithelial dome, where the majority of DCs reside[45].

Upon antigen or bacteria internalization, DCs undergo maturation before migrating to draining lymph nodes, where they activate and influence functional differentiation of naïve T-cells. In accordance to studies in experimental colitis[46], we were the first to show that also in severe AP, DCs internalize bacteria and migrate away from the SED.

It has been widely discussed that the delicate balance between Th1 and Th2 is pivotally controlled by DCs. The ability of DCs to modulate T-cell activity is attributable to their expression of cytokines and co-stimulatory molecules that is upregulated during maturation. Discriminative factors in this respect are IL-12 levels and expression of CD86 both contributing to a more pro-inflammatory Th1 response, while IL-10 suppresses the effects of IL-12[47]. We observed in animals subjected to AP, a higher rate of DCs that express the co-stimulatory molecule

40 Ammori, 2003
41 Pulendran *et al.*, 2001
42 Neutra *et al.*, 2001
43 Gebert, 1997
44 Rescigno *et al.*, 2001
45 Neutra *et al.*, 2001
46 Silva *et al.*, 2006
47 Kronin *et al.*, 2000

DENDRITIC CELLS PLAY A PIVOTAL PART IN THE INITIATION AND ORCHESTRATION OF MUCOSAL IMMUNE RESPONSES

ALSO IN
SEVERE ACUTE
PANCREATITIS
DENDRITIC CELLS
INTERNALIZE
BACTERIA AND
MIGRATE AWAY
FROM THE
SUB-EPITHELIAL
DOME

CD86 and produce IL-12, which are both strong Th1 skewing signals. Moreover, after induction of AP, elevated levels of IL-12 were detected in MLNs.

After activation and differentiation of the T-cells in MLNs, DCs also take part in programming T-cells to home-back to the intestinal tissue[48], where Th2-cells, in the presence of IL-12 suppressing factors, maintain their phenotype. However when surrounded by high levels of IL-12, Th-2 cells abandon their phenotype and will adopt a more Th-1 like phenotype which will only further enhance the inflammatory response[49]. In that respect it is even more interesting that the current data show high levels of mucosal IL-12. This indicates that the mucosal environment after induction of AP is not conductive to a Th2 response, increasing the risk of an exaggerated Th1 immune response and exacerbating mucosal barrier dysfunction[50]. In order to break this vicious circle, it would be beneficial to create an environment more conductive to Th2 polarized immunity with high IL-10 levels to prevent Th2 cells from changing phenotype and executing Th1 like responses.

Numerous investigations have been conducted on the immunomodulatory effects of probiotics. Lactobacilli and other commensal bacteria appear to affect immunity at the innate as well as humoral and cellular levels. It seemed therefore plausible that DCs play a central role in mediating effects of probiotics[51]. It is widely known that microbial products play a key role in regulating DC responses. DCs interact with microbes using toll-like receptors (TLR), which makes them able to interpret signals from different microbes, and instruct the adaptive immune response to execute distinct patterns of immunity. DCs inhabiting the gut are mostly immature and thus potentially prone to differentiation by the environment, which contains abundance of microorganisms. And indeed after modulation of the microbiotia by probiotic administrations, DCs in AP-subjected animals, did not express the same high levels of CD86 and IL-12 as compared to placebo treated animals. This is in accordance with previously published work, showing that *Lactobacillus reuteri* is effective in downregulating CD86 and IL-12 expression on bone marrow derived DCs[52]. Furthermore, pretreatment with probiotics decreased AP-induced elevation in IL-12 levels both in Peyer's patches and in MLNs, suggesting that treatment with probiotics is able to shape the local environment towards a more Th2 conductive setting, putatively by modulating DC maturation. This may be crucial in the course of severe AP, as IL-12-induced IFN-γ will only further deteriorate the already failing mucosal barrier[53].

Previous work by our group has shown that the same combination of probiotics as employed in this study, induces high levels of IL-10 in peripheral blood mononuclear cells[54]. The current data are in line with these results showing that pretreatment with probiotics increases IL-10 levels in Peyer's patches and in MLN, even compared with healthy control animals. As IL-10 is known to suppress IL-12 production and consequently IFN-γ expression, the increased IL-10 levels found are yet another indicative finding that pretreatment with probiotics may create a

48 Stagg *et al.*, 2002
49 Kalinski *et al.*, 2000
50 Beaurepaire *et al.*, 2009
51 Marteau, 2000
52 Christensen *et al.*, 2002
53 Beaurepaire *et al.*, 2009
54 Timmerman *et al.*, 2007

mucosal environment that is supportive of a Th2 response. Furthermore, IL-10 downregulates antigen presentation by translocation of MHC class II complexes[55] and acts on macrophages to prevent their activation of pro-inflammatory molecules and chemokines, thus inhibiting T-cell recruitment back into the intestine[56]. Taken together, sufficient IL-10 levels and reduced levels of IL-12 both mucosally as well as in the MLNs may be key factors in protecting the mucosal barrier from exaggerated immune responses and subsequent barrier failure. And indeed, the study herein shows that AP-induced mucosal barrier dysfunction was ameliorated by pretreatment with probiotics. Additionally, probiotics reduced bacterial adherence, which is the first step in colonization and penetration of bacteria[57].

It is widely assumed that microbial products are able to influence maturation and function of DCs. Detailed mechanistic factors, however still need to be elucidated. Previous work has shown that individual probiotic bacteria displayed different effects on DC cytokine production, arguing against a common bacterial component such as peptidoglycan mediated effects. Furthermore, there is evidence that different subsets of DCs react broadly similar to bacterial products despite the fact that they are known to express a different arrays of TLRs[58], indicating that other factors may also be involved in differentiation of DC function. A candidate factor that modulates DC function in this respect may be activation of PPAR-γ. PPAR-γ belongs to a family of nuclear receptors and is known to exert anti-inflammatory effects by interfering with inflammatory transcription factors. It is accepted that PPAR-γ is a negative regulator of macrophage activation, and inhibits production of monocyte inflammatory cytokines[59]. Furthermore, activation of PPAR-γ has been shown to reduce upregulation of co-stimulatory molecules and IL-12 production in LPS stimulated DCs and therefore preventing maturation of DCs[60]. Moreover, PPAR-γ seems to be involved in the antigen presenting capacity as PPAR-γ activation in DCs reduced their T-cell priming ability[61]. These investigations are in line with the negative correlation between mucosal PPAR-γ activation and Il-12 levels found in this study. But most interestingly, probiotic pretreatment resulted in enhanced positive double staining for PPAR-γ and CD103, suggesting that probiotics increase PPAR-γ levels in mucosal DCs, which has not been previously reported.

PPAR-γ can be activated by numerous dietary compounds such as polyunsaturated fatty acids or eicosanoids[62] and recently also probiotics have been identified as possible means of PPAR-γ activation. Only little is known about the precise mechanism by which probiotics activate PPAR-γ. One possible explanation is the ability of lactic acid bacteria to produce conjugated linoleic acid, which has been shown to exert PPAR-γ activating properties[63]. In a very recent study, however it was shown that probiotics may produce and employ H_2O_2 as a molecular signal

55 De Smedt *et al.*, 1997
56 Asseman *et al.*, 1999
57 Lu & Walker, 2001
58 Hart *et al.*, 2004
59 Ricote *et al.*, 1998
60 Faveeuw *et al.*, 2000
61 Klotz *et al.*, 2007
62 Marion-Letellier *et al.*, 2009
63 Ewaschuk *et al.*, 2006

transducer to induce PPAR-γ[64]. This finding is especially interesting in the light of a recently published study in severe AP patients[65]. This randomized controlled trial showed an increased incidence of intestinal ischemia after probiotic treatment, indicating that in patients that are in a critically ill state and whose redox system is already critically imbalanced, treatment with probiotics is at this moment not defendable. Therefore, to safely benefit from this mechanism of action of probiotics, a preventive approach seems to be a safer option, such as maintaining remission in inflammatory bowel disease in which probiotics have already shown positive results[66]. In addition, patients with ulcerative colitis have been shown to have a reduced expression of PPAR-γ, particularly in colonic epithelial cells, without any mutation in the PPAR-γ gene[67], while in human Crohn's disease, the gene encoding PPAR-γ (ppar γ) has been identified as a susceptibility gene[68].

It should be noted that it has been previously reported that effects of probiotics on DCs are strain dependent[69] and that based on the current data it is impossible to determine strain specificity or synergistic effects in the combination of probiotics. Further studies are needed to elucidate these effects. However, as Th2 polarizing factors share the property of being IL-12 suppressors, the net result of multispecies treatment is likely to favor a Th2 response. This has been shown in *in vitro* experiments on bone marrow derived DCs in which the effects of otherwise IL-12 inducing strains was inhibited by co-treatment with IL-10 inducing strains, without inhibitory effects on IL-10 production[70].

In conclusion, our data are the first to describe that AP induces bacterial adherence to FAE, accompanied by increased uptake of bacteria into DCs, which in turn causes maturation and migration of DCs away from the SED and which eventually leads to a Th1 type immune response. Furthermore, these data indicate that in AP subjected animals, pretreatment with the studied probiotics modulates DC maturation and cytokine expression from an inflammatory towards a regulatory profile, putatively via upregulation of PPAR-γ in mucosal DCs. Altering mucosal inflammation by activation of PPAR-γ induced by probiotics may have implications in treatment strategies for inflammatory bowel disease. Therefore further elucidation of mechanisms by which probiotics modulate the mucosal immune system may facilitate development of individually tailored and disease specific therapeutic supplements.

64 Voltan *et al.*, 2008
65 Besselink *et al.*, 2008
66 Gionchetti *et al.*, 2000
67 Dubuquoy *et al.*, 2003
68 Sugawara *et al.*, 2005
69 Christensen *et al.*, 2002
70 Christensen *et al.*, 2002

9

Role of mast cells and PPAR-γ: Effects of probiotics on chronic stress-induced intestinal permeability in rats.

Probiotics; the role of mast cells and PPAR-γ

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ABSTRACT

BACKGROUND

Chronic stress, which may be involved in the clinical course of inflammatory and functional bowel diseases, disrupts intestinal barrier function by routes involving mast cells. Probiotics have been shown to ameliorate the deleterious effects of stress on intestinal function, but mechanisms remain to be elucidated. PPAR-γ[1] is activated as an endogenous defense mechanism during chronic stress and evidence suggests that probiotics reduce the degradation of PPAR-γ. As a source of the endogenous agonist for PPAR-γ, 15d-PGJ_2, and as an important mediator of the stress response, mast cells may have both a beneficial and a deleterious role in the effects on intestinal function by probiotics.

AIM

Our aim was to study if mast cells contribute to the positive effects of probiotic therapy on intestinal function in a rat model of chronic stress.

METHODS

32 Mast cell deficient (Ws/Ws) and 32 wild-type (+/+) rats were subjected to water avoidance stress (WAS) or sham stress (SS) 1hr/day for 10 days. Seven days prior to the onset of stress, probiotics (PB)[2] were added to the standard diet (St) in half of the animals.

1 Peroxisome proliferator-activated receptor-γ
2 Multispecies combination of 10 different lactic acid bacteria, Ecologic® 825

To determine dependence of PPAR-γ an additional 8 WAS subjected, probiotic-fed wild-type rats were injected daily with the specific PPAR-γ antagonist T0070907. The colonic mucosa was exposed to *E. coli* HB101 incorporated with green fluorescent protein and permeability was assessed in Ussing chambers. Mesenteric lymph nodes (MLN) were cultured to determine bacterial translocation.

RESULTS

As expected, chronic stress induced a marked increase in ileal permeability to *E. coli* HB101 in wild-type rats[3]. This breach in barrier integrity was less pronounced in Ws/Ws rats[4]. Probiotics prevented stress-induced effects only in wild-type rats[5]. Furthermore, only in the presence of mast cells did probiotics reduce the enhanced bacterial translocation to MLNs during chronic stress. In wild-type rats treated with a PPAR-γ antagonist, the barrier protective effects of probiotics were diminished.

CONCLUSIONS

Mast cells acting via a PPAR-γ dependent pathway contribute to the beneficial effects of probiotics on chronic stress-induced mucosal dysfunction in rats.

3 0.17 ± 0.1 x10^3CFU/hr in SS/St/++ *vs.* 2.13 ± 0.4 in WAS/St/++; $P<0.001$

4 2.13 ± 0.4 x10^3CFU/hr in WAS/St/++ *vs.* 1.19±0.3 in WAS/St/WsWs; $P<0.01$

5 82% decrease in +/+ *vs.* 0.0% in Ws/Ws rats in *E. coli* HB101 passage

INTRODUCTION

The relationship between psychosocial stress and several gastrointestinal disorders is widely accepted. Stress influences subjective perception of gastrointestinal symptoms by patients[6] and has been shown to trigger the onset and modify the clinical course of certain gastrointestinal diseases[7]. For instance, inflammatory activity of ulcerative colitis and the risk of disease exacerbation has been associated with stress[8].

It has previously been reported that psychological stress causes disruption of the epithelial barrier[9], bacterial adherence and mucosal inflammation[10]. Experimental studies utilizing a rat model of chronic stress have shown that these deleterious effects of stress are mediated via mast cells[11].

A question of special relevance is the possibility that some of the changes observed in stress would be a feature not of the deleterious effects of stress but of some reversible type of adaptive plasticity[12]. Most species of higher multicellular organisms have evolved complex homeostatic and defense mechanisms that allow cells to overcome stressful stimuli. One such mechanism that has received considerable attention is peroxisome proliferator-activated receptors (PPARs), a group of 3 ligand-regulated transcription factors. One of its isoforms, PPAR-γ, exerts anti-inflammatory effects by interfering with the activity of inflammatory transcription factors, such as Activator Protein-1 (AP-1), Signal Transducers and Activators of Transcription (STAT), and nuclear factor-κB (NF-κB)[13]. PPAR-γ has a high-affinity ligand, being 15-deoxy-Δ prostaglandin J_2 (15d-PGJ_2)[14], which is derived from PGD_2 and mainly released by mast cells[15]. Activation of PPAR-γ by endogenous agonists, such as 15d-PGJ_2, or synthetic agonists can be considered as a pathway to halt the inflammatory response. Indeed, PPAR-γ ligands have been shown to reduce colonic inflammation in both dextran sodium sulfate and trinitrobenzene sulfonic acid colitis models in mice[16]. Furthermore, up-regulation of intestinal PPAR-γ expression occurs in response to LPS challenge[17] and stress-induced colonic inflammation[18] suggesting that the enhanced expression of PPAR-γ in the colon may occur as a compensatory mechanism to down-regulate mucosal inflammation.

Endogenous probiotic bacteria of the gut such as *Bifidobacterium* and *Lactobacillus* play a vital role in maintaining the intestinal mucosal barrier[19]. Probiotic bacteria have been shown to modulate intestinal epithelial barrier function and cytokine secretion through effects on epithelial cells and modulation of the NF-κB and

6 Dickhaus *et al.,* 2003
7 Wilhelmsen, 2000
8 Duffy *et al.*, 1991; Levenstein *et al.*, 2000
9 Saunders *et al.*, 1994; Kiliaan *et al.*, 1998; Santos *et al.*, 1999; Santos *et al.*, 2000
10 Soderholm *et al.*, 2002
11 Santos *et al.*, 2001
12 McEwen, 1998
13 Ricote *et al.*, 1998
14 Kliewer *et al.*, 2001
15 Lewis *et al.*, 1982
16 Su *et al.*, 1999; Desreumaux *et al.*, 2001
17 Ewaschuk *et al.*, 2007
18 Ponferrada *et al.*, 2007
19 Madsen *et al.*, 2001; Garcia-Lafuente *et al.*, 2001

MOST
HIGHER SPECIES
HAVE EVOLVED
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STRESSFUL
STIMULI

WE HYPOTHESIZED THAT MAST CELLS CONTRIBUTE TO THE POSITIVE EFFECTS OF PROBIOTIC THERAPY ON INTESTINAL FUNCTION DURING CHRONIC EXPERIMENTAL STRESS

PPAR-γ pathways[20]. For example, Ewaschuk *et al.* (2007) showed that probiotics attenuate intestinal barrier dysfunction in a LPS-induced mouse model of sepsis via PPAR-γ dependent pathways, which was proven by the fact that beneficial effects of probiotics were abolished by PPAR-γ inhibition.

The activity of PPAR-γ is balanced by ligand binding on one hand and the degradation of PPAR-γ on the other, of which the latter occurs through the ubiquitin-proteasome system[21]. It has previously been shown that probiotics reduce cellular proteasomal activity[22]. Therefore, it is possible that the maintenance of PPAR-γ activity is due to a probiotic-induced reduction in PPAR-γ degradation.

Given the ability of mast cells to produce the endogenous agonist for PPAR-γ, 15d-PGJ_2, we hypothesized that mast cells contribute to the positive effects of probiotic therapy on intestinal function during chronic experimental stress.

20 Madsen *et al.*, 2001; Lutgendorff *et al.*, 2008; Jijon *et al.*, 2004; Gionchetti *et al.*, 2005
21 Clark, 2002
22 Jijon *et al.*, 2004; Petrof *et al.*, 2004

MATERIALS AND METHODS

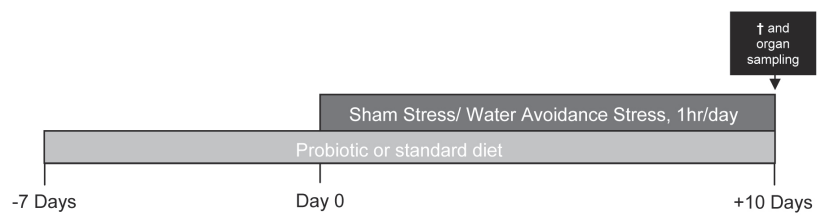
RATS

Mast cell-deficient Ws/Ws rats and their +/+ littermate controls (SLC Japan, Shizuoka, Japan) were maintained under constant housing conditions (temperature (22°C), relative humidity (60%) and a 12-hour light/dark cycle) and had free access to water and standard rat chow throughout the experiment. Ws/Ws rats have a 12-base deletion in the tyrosine kinase domain of the c-*kit* gene, and, by 10 weeks of age, they show significantly reduced numbers of mast cells in their intestine, whereas +/+ rats have a normal number of mast cells[23]. Sixty-four weight and age matched rats were acclimatized for two weeks by daily handling prior to the start of chronic psychological stress and randomly allocated into eight experimental groups (n=8): 1)+/+ rats; sham stress and basal diet 2) +/+ rats; chronic stress and basal diet 3) +/+ rats; sham stress and probiotic diet 4) +/+ rats; chronic stress and probiotic diet 5) Ws/Ws rats; sham stress and basal diet 6) Ws/Ws rats; chronic stress and basal diet 7) Ws/Ws rats; sham stress and probiotic diet 8) Ws/Ws rats; probiotics chronic stress and probiotic diet. To determine dependence of PPAR-γ, an additional 8 stressed, probiotic-fed wild-type rats were injected daily with the specific PPAR-γ antagonist, T0070907 (120μg/kg, i.p.). The drug was injected 1 hr before the onset of the stress. The experimental design (fig 1) was approved by the local committee of animal ethics.

FIGURE 1

Experimental design

Wild-type and mast cell deficient rats were handled daily for two weeks before the start of the experiment. Probiotics were added to drinking water seven days prior to the start of daily exposure to sham stress or water aversion stress. After 10 days of sham or water avoidance stress all animals were anesthetized, sacrificed and organs of interest were harvested.



23 Morimoto *et al.*, 1993

PROBIOTICS AND PLACEBO

The study product (*Ecologic® 825*, Winclove Bio Industries, Amsterdam, The Netherlands) consisted of nine viable and freeze-dried probiotic strains; *Bifidobacterium bifidum* (W23), *Bifidobacterium lactis* (W51), *Bifidobacterium lactis* (W52), *Lactobacillus acidophilus* (W22), *Lactobacillus casei* (W56), *Lactobacillus paracasei* (W20), *Lactobacillus plantarum* (W61), *Lactobacillus salivarius* (W24), *Lactococcus lactis* (W19.) The probiotic mixture was rehydrated to a concentration of 10^9 CFU/ml in sterile distilled water. Separate groups of rats were fed a standard diet and sterile water throughout the study (basal diet) and the other groups received the same diet supplemented with probiotics added to drinking water, which was changed daily, for seven days prior to the start of chronic psychological stress (probiotic diet) and continued to receive probiotics until the experiments were concluded. Both groups were provided access to food and water *ad libitum*.

CHRONIC WATER AVOIDANCE STRESS (WAS)

Rats from both the basal and probiotic diet groups were handled daily for 2 weeks and then subjected to either WAS or sham stress for one hour/day for 10 days, as previously described[24]. Briefly, rats were weighed and then placed onto a platform (h=8, Ø=6 cm) affixed to the centre of a plastic container (h=56, Ø=50 cm) filled with warm water (25°C) to 1 cm below the platform. Rats subjected to sham stress were placed on the same platform in a container without water. The procedures were performed between 8:00 and 10:00 AM to minimize the effect of circadian rhythm. Previously, we determined that the effect of stress on epithelial function was maximal several hours after stress[25]. Based on this finding, 4 hours after the last WAS or SS session, the rats were anaesthetized by isoflurane inhalation and blood was collected for subsequent analysis of serum corticosterone. Mesenteric lymph nodes (MLNs) were removed aseptically to determine bacterial translocation. Intestinal segments from the distal colon (starting at the promontory) were taken for permeability studies and determination of bacterial adherence.

FUNCTIONAL STUDIES

Growth. Rats were weighed daily. Change in weight was calculated and expressed percentage of initial body weight.

Serum corticosterone. Total corticosterone in blood samples was analyzed using the IDS OCTEIA corticosterone kit (IDS, Boldon, U.K) according to the manufacturer's instructions. All samples were run in duplicate and absorbances were measured at 450 nm, with a reference wavelength of 650 nm, in an ELISA microtitre plate reader (Anthos htIII; Anthos, Labtec Instruments, Salzburg, Germany). Corticosterone levels are expressed in ng/ml.

Defecation. Defecation, as an indirect index of colonic propulsive activity[26] was determined at each stress session. Pellets expelled during the 1-hour daily stress/sham periods were counted and expressed as the number of fecal pellets/h.

24 Soderholm *et al.*, 2002
25 Soderholm *et al.*, 2002
26 Barone *et al.*, 1990

USSING CHAMBER EXPERIMENTS

Mucosal permeability was measured as previously described[27]. In brief, intestinal specimens were stripped of the external muscle and myenteric plexus while immersed in ice cold oxygenated Krebs, and were mounted into modified Ussing chambers (Harvard apparatus Inc., Holliston, Massachusetts, USA)[28]. A tissue surface area of 9.6 mm^2 was exposed to 3 ml of circulating Kreb's buffer which was oxygenated and maintained at 37°C. To provide energy to the tissue, the serosal buffer contained 10 mM glucose which was osmotically balanced by 10 mM mannitol in the mucosal buffer. The chambers contained agar-salt bridges to measure the potential difference across the tissue, to monitor tissue viability. Tissue was clamped periodically at a zero voltage by introduction of an appropriate short circuit current (Isc) with an automated voltage clamp (World Precision Instruments, Sarasota, Florida, USA). Tissue conductance, representing paracellular permeability to ions, was calculated by Ohm's law. Baseline values for Isc, indicating net ion secretion, and conductance, as an inverse measure of tissue resistance, were recorded at equilibrium, 40 minutes after mounting of the intestinal segments.

Mucosal to serosal transport of macromolecules was assessed by measuring the inert paracellular permeability probe ^{51}Cr-EDTA, (MW 384 D; Perkin-Elmer, Boston, MA, USA). ^{51}Cr-EDTA was added to a final concentration of 4 μCi/ml. Serosal samples (300 μl) were collected at 0, 30, 60, 90 and 120 minutes after start and transepithelial permeability to ^{51}Cr-EDTA was determined as previously described[29]. Permeability was calculated in 3 ileal and colonic samples per animal during 30–120 minutes for both markers.

To assess intestinal permeability to live bacteria 3 samples per rat were mounted in Ussing chambers. Live, green fluorescent protein (GFP)-incorporated *Escherichia coli* HB 101 were added, after equilibration, to the mucosal side of the reservoirs at a final concentration of $1x10^8$ CFU/ml. After 120 minutes, the entire volume of the serosal compartments was collected and analyzed at 488 nm in a fluorimeter (Cary Eclipse, Varian, Victoria, Australia) where one unit refers to 1.5 $x10^3$ CFU/ml.

BACTERIAL ADHERENCE AND PENETRATION

To measure bacterial translocation across the intestinal epithelium, MLNs were harvested aseptically, weighed and homogenized in sterile Triton X-100 (0.1%). To determine bacterial adherence, colon samples of 1 cm were excised, weighed and washed thoroughly in sterile saline. Pieces were then homogenized in sterile Triton X-100 (0.1%) and diluted serially. Homogenates were spread on blood agar plates (PML Microbiologicals, Mississauga, ON, Canada) and incubated at 37°C for 24 h. To study *Lactobacillus* species specifically, samples were plated on deMan Rogosa Sharpe (MRS; EMC Chemicals, Gibbstown, NJ, USA) agar plates and incubated anaerobically at 37°C for 48 h. Colonies were then counted and expressed as colony-forming units per gram of tissue (CFU/g).

27 Lutgendorff *et al.*, 2009
28 Grass & Sweetana, 1988
29 Lutgendorff et al., 2009

MYELOPEROXIDASE ACTIVITY

Mucosal inflammation was assessed by measuring mucosal activity of myeloperoxidase (MPO), an enzyme found in granulated cells and previously shown to be a reliable index of inflammatory activity[30]. Muscle-stripped mucosal tissues were snap frozen and stored at -80°C until analyses were performed. Frozen samples were submerged in liquid nitrogen, before being pulverized and weighed. Pulverized material was homogenized in 0.5% hexadecyltrimethyl-ammonium bromide (HTAB) in 50 mM potassium phosphate buffer, pH 6.0, to provide a 10% homogenate (w/v). Samples were freeze-thawed and sonicated on ice. Suspensions were centrifuged at 14.000×g for 15 minutes. The detection of MPO was based on its ability to degrade hydrogen peroxide. The MPO activity was determined by mixing 50 μL of supernatant with 1.45 mL of 50 mM potassium phosphate buffer containing 0.167 mg/mL of O-dianisidine hydrochloride. The reaction was started by addition of hydrogen peroxide to a final concentration of 0.0005% and was measured spectrophotometrically. Change in absorbance at 460 nm was measured every 15 s for 5 min using a Genesys 10 UV spectrophotometer from Thermo Spectronic (Rochester, NY, USA). The MPO activity is expressed as U/mg tissue (1 U is the quantity of MPO required to convert 1μmol of H_2O_2 into water in 1 minute at 22°C).

15-DEOXY-Δ PROSTAGLANDIN J_2 LEVELS

Intestinal 15d-PGJ_2 levels were determined using a enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA). Briefly, tissue was homogenized and after centrifugation (10,000x*g*, 10min, 4°C) protein contents were measured using Bradford's method[31]. Quantification was performed following the manufacturer's instructions and using the supplied standard. Results are expressed as pg/mg protein.

PPAR-γ ACTIVATION

To determine activated PPAR-γ, PPAR-γ transcription factor levels were determined in the nuclear fraction of tissue homogenates. Nuclear extraction was performed as previously described by our group[32] and PPAR-γ transcription factor levels were quantified using a rat PPAR-γ transcription factor ELISA kit (USCN LIFE, Missouri, TX, USA) as indicated by the manufacturer. Results were normalized to nuclear protein and are expressed as ratio to controls.

RAT MAST CELL PROTEINASE II.

Rat mast cell proteinase (RMCP)-II was determined in colonic homogenates by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Moredun Scientific Ltd., Midlothian, Scotland). The RMCP-II content of colonic mucosa was expressed as ng/mg protein.

30 Smith & Castro, 1978
31 Bradford, 1976
32 Trulsson *et al.*, 2004

STATISTICAL ANALYSIS

All data were assessed for normal distribution using Shapiro–Wilk's test. Parametric values are presented as mean (SEM). Statistical analysis was performed by ANOVA followed by Tukey's HSD test for multiple comparisons (SPSS 13.0 Inc, Chicago, Illinois, USA). Non-parametric values are given as median (25-75th inter-quartile range). Comparisons were done by Kruskal-Wallis when more than two groups were compared. Mann-Whitney U test was used to compare two groups. Spearman's rank correlation coefficients were computed for correlation analyses. *P* values <0.01 were considered significant.

RESULTS

CHRONIC STRESS INDUCES SYSTEMIC CHANGES IN BOTH +/+ AND WS/WS RATS REGARDLESS OF DIET

None of the rats receiving probiotics showed clinical signs of distress throughout the pretreatment period. During this 7 day period, animal weight increased continuously and was equal in all groups (fig 2A). When exposed to stress, weight gain was absent in both +/+ and Ws/Ws and animal weight remained consistent during the 10-day stress period. All sham stress animals, however continued to gain weight during the experimental period. Treatment with probiotics did not affect animal weight.

As expected, in all animals under chronic stress, serum corticosterone levels were significantly increased. Control +/+ rats had an average serum corticosterone level of 61.7 (3.5) which reached double values in chronically stressed rats[33]. Serum corticosterone levels were similarly elevated after a chronic stress period in Ws/Ws rats[34]. A diet supplemented with probiotics had no effects on serum corticosterone values (data not shown).

Propulsive activity as measured by the number of fecal pellets that was expelled during the daily stress sessions was enhanced during chronic stress (fig 2B). Probiotic pretreatment ameliorated this stress-induced elevation in propulsive activity.

PROBIOTICS MAINTAINED COLONIC BARRIER FUNCTION AND REDUCED BACTERIAL TRANSLOCATION

In +/+ wild-type rats, a 10-day stress period resulted in a marked mucosal barrier dysfunction, via both transcellular and paracellular passage routes. Colonic barrier function was assessed in Ussing chambers by determination of baseline conductance, representing paracellular ion flux and permeability to ^{51}Cr-EDTA, and live *E. coli* HB101. As shown in fig 3, chronic stress significantly increased baseline conductance and permeability to ^{51}Cr-EDTA (fig 3A, B) both indicative of a breakdown in mucosal barrier through paracellular pathways. Probiotic treatment markedly ameliorated the stress-induced increase in paracellular permeability. Rats treated with probiotics were also less susceptible to stress-induced increased passage of bacteria (fig 3 C). Increased intestinal permeability to bacteria also showed in increased stress-induced bacterial translocation to MLNs. One hundred percent of rats, subjected to chronic stress demonstrated bacterial translocation to MLNs (table 1). Probiotic treatment reduced bacterial translocation: 1 of the 8 rats receiving probiotics had culturable bacteria in the MLNs, and furthermore, bacterial numbers were significantly reduced. No bacteria were found to be culturable on anaerobically incubated deMan Rogosa Sharpe plates, suggesting that no culturable lactobacillus species translocated to MLNs (data not shown). In contrast to colonic permeability, elevation in ion secretion was not affected by addition of probiotics into the drinking water of chronically stressed rats (fig 3D)

33 137.6 (11.0); $P<0.001$

34 SS 68.3 (6.8) *vs.* WAS 139.0 (8.5); $P<0.001$

Increased bacterial adherence, which is the first step in colonization and penetration of bacteria[35], is known to be increased upon chronic stress[36]. Stress induced bacterial adherence of potential pathogens was only mildly reduced after probiotic treatment (fig 4A). Interestingly, treatment with probiotics prevented the deleterious effects of chronic stress on mucosa-associated *lactobacillus* species, which were diminished after 10 days of stress (fig 4B).

PROBIOTICS PREVENTED STRESS-INDUCED INCREASE IN MYELOPEROXIDASE ACTIVITY

Increased invasion of bacteria and antigens into the subepithelial layer is known to initiate a mucosal inflammatory reaction and chronic stress have been associated with a mild degree of inflammation in the mucosa[37]. To determine if, in addition to the attenuation of stress-induced barrier dysfunction, probiotics were effective in amelioration of the mucosal inflammatory response, MPO activity was quantified. Chronic stress caused enhanced MPO activity. In contrast, colonic tissue from rats pretreated with probiotics did not demonstrate an increase in MPO activity in response to stress, nor did probiotic treatment on its self cause an elevation in MPO activity (fig 5).

ROLE OF MAST CELLS

Mast cells are known to play a pivotal role in stress-induced effects on the intestinal barrier, and therefore mucosal RMCP-II levels, which is indicative of activation of mast cells[38] were assessed. Wild–type +/+ rats subjected to chronic stress demonstrated significantly elevated levels of RMCP-II in comparison with sham stressed rats (fig 6). Probiotic treatment of rats before and during exposure to stress resulted in a decrease in mucosal RMCP-II levels, possibly because treatment with probiotics modulated degranulation patterns of mast cells. Ws/Ws rats demonstrated a small, but significant increase in RMCP-II in response to stress (non detectable level in sham stressed rats *vs.* 0.37 (0.012) ng/mg protein after chronic stress; $P<0.01$), indicating that Ws/Ws rats, however mast cell deficient, still have a small activity of mast cells, which has also been shown previously[39].

To determine the role of mast cells in probiotic-induced amelioration of intestinal barrier dysfunction during stress, Ws/Ws mast cell deficient rats were pretreated with probiotics and colonic barrier dysfunction and mucosal inflammation in response to stress were assessed. First off, chronic stress induced mucosal barrier dysfunction in Ws/Ws rats, however not to the same extend as in wild-type +/+ rats. As shown in fig 7, probiotic treatment did not affect the stress-induced mucosal barrier dysfunction in Ws/Ws rats. In addition, stress-induced mucosal inflammation was unchanged by probiotic treatment in Ws/Ws rats (fig 7D). These data suggest that probiotics exert their protective effects on barrier function via a mast cell dependent pathway in this rat model of chronic stress.

35 Lu & Walker, 2001
36 Soderholm *et al.*, 2002
37 Soderholm *et al.*, 2002
38 Woodbury *et al.*, 1984
39 Arizono *et al.*, 1993

ROLE OF PPAR-γ IN STRESS-INDUCED BARRIER DYSFUNCTION

Activation of PPAR-γ has been shown to play a role in the maintenance of mucosal barrier function and to attenuate inflammatory responses[40]. Ten days of exposure to chronic stress resulted in enhanced levels of PPAR-γ transcription factor in colonic mucosa in wild-type rats (fig 8A). In mast cell deficient animals this stress-induced increase in mucosal PPAR-γ transcription factor was absent. Probiotic treatment substantially increased levels of PPAR-γ transcription factor in both sham stressed and chronically stressed wild-type rats. That in contrasts to Ws/Ws animals in which probiotic treatment resulted in a mild, however significant, increase in PPAR-γ transcription factor level, which was not further enhanced after exposure to stress.

To determine if this modulation in PPAR-γ was a reflection of enhanced endogenous ligand activity, 15d-PGJ_2 was measured. Addition of probiotics to drinking water enhanced mucosal 15d-PGJ_2 levels, however only in wild-type animals exposed to stress, suggesting probiotic-induced enhanced levels of 15d-PGJ_2 and thereby increased PPAR-γ activation, is mast cell dependent in this model of chronic stress.

To test if the effects of probiotics on intestinal barrier function and mucosal inflammation were indeed PPAR-γ depended, eight wild-type probiotic fed rats were treated with a selective PPAR-γ antagonist, T0070907. As shown in fig 9, inhibition of PPAR-γ abrogated the ability of probiotics to protect against stress-induced barrier dysfunction and mucosal inflammation. Of note, animals injected with T0070907 still demonstrated elevated levels of 15d-PGJ_2 after exposure to stress and treatment to probiotics (fig 9E).

40 Su *et al.*, 1999; Ponferrada *et al.*, 2007

TABLE 1

Bacterial translocation to mesenteric lymph nodes

Group	No of rats affected/ total no of rats in group	Median (range) CFU/gram tissue
Sham stress, standard diet (n=8)	0/8	0
WAS, standard diet (n=8)	8/8	$1.4x10^3$ ($2.3x10^2$-$2.3x10^4$)*
Sham stress, probiotic diet (n=8)	0/8	0
WAS, probiotic diet (n=8)	1/8	0 (0-$1.2x10^2$)

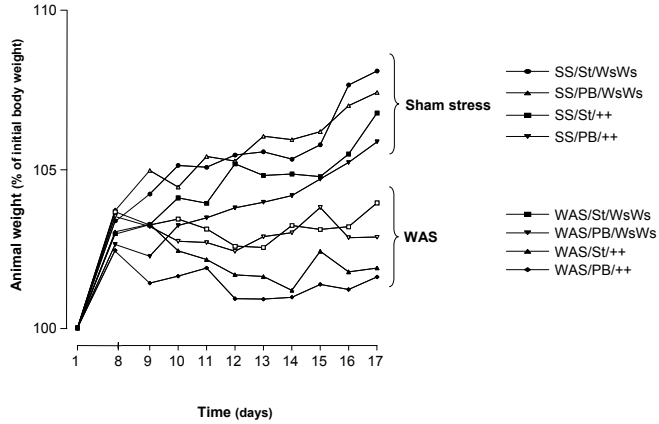
Rats were subjected to water avoidance stress (WAS) or sham stress for 10 days, for one hour/day. Homogenates of mesenteric lymph nodes (MLN) from the ileocaecal and left colonic regions of each rat were cultured and incubated for two days at 37°C. Bacterial translocation data are represented as median (range) of the total CFU (both aerobic and anaerobic) cultured from MLN of each rat after 48 hours of incubation on blood agar plates.
*P<0.01 in comparison with sham stress or probiotic treated WAS animals.

FIGURE 2

Stress prevents normal weight gain and increases propulsive activity

(A) This figure shows the weight development of the rats during the course of the study. Mast cell deficient rats and their normal +/+ litter mates were pretreated with probiotics (PB) or a standard diet (St) and were submitted to sham stress (SS) or water aversion stress (WAS) for one hour/day on 10 consecutive days. Change in body weight is expressed as percentage of initial body weight on day one. (B) Fecal pellet expulsion during each of the 10 stress sessions was quantified to assess propulsive activity. Comparisons were preformed using ANOVA followed by Tukey's HSD.

A



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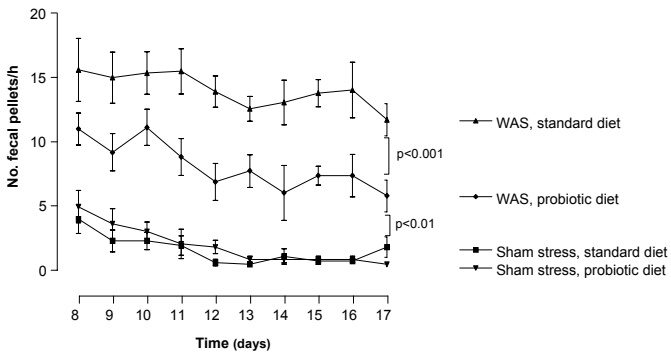


FIGURE 3

Probiotics decrease stress-induced barrier dysfunction in colonic mucosa

After seven days of standard diet (n=8) or probiotics (n=8) wild type +/+ rats were subjected to water avoidance stress (WAS) (n=8) or sham stress (n=8) for 10 consecutive days. Colonic mucosa was mounted in Ussing chambers. In wild-type control rats stress induced (A) an increase in base line conductance and (B) increased permeability to ^{51}Cr-EDTA. This coincided with increased permeability to (C) live fluorescent *E. coli* HB101. Probiotic administration prevented stress induced changes in all parameters except for (D) short circuit current. Graphs show means ± SEM. Data were collected from independently acquired sets of three tissue segments per rat. Comparisons were preformed using ANOVA followed by Tukey's HSD.

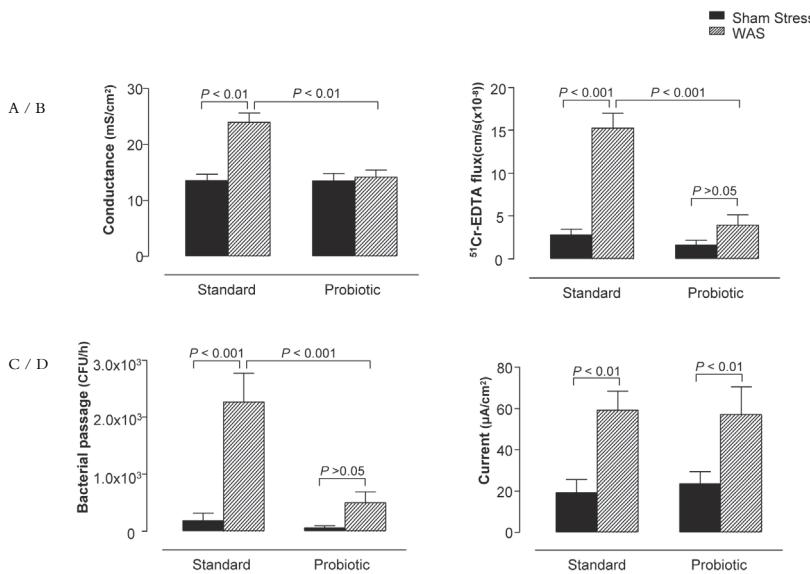


FIGURE 4

Probiotic-induced changes in bacterial adherence

After seven days of standard diet (n=8) or probiotics (n=8) wild type +/+ rats were subjected to water avoidance stress (WAS) (n=8) or sham stress (n=8) for 10 consecutive days. Colonic samples were thoroughly rinsed in sterile PBS, homogenized and plated on (A) blood agar plates to determine the adherence of potential pathogens to epithelium. (B) To quantify *lactobacillus* species that associated with colonic mucosa, colonic homogenates were plated on deMan-Rogosa Sharpe plates. Graphs show mean ± SEM on a logarithmic scale. Comparisons were preformed using ANOVA followed by Tukey's HSD.

A / B

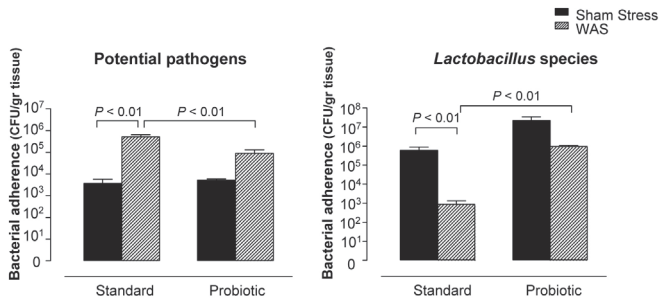


FIGURE 5

Probiotics reduce stress-induced mucosal inflamation

After seven days of standard diet (n=8) or probiotic (n=8) wild type +/+ rats were subjected to water avoidance (WAS) stress or sham stress for 10 consecutive days. Myeloperoxidase activity (MPO) was determined in colonic mucosa. Graph shows mean ± SEM. Comparisons were preformed using ANOVA followed by Tukey's HSD.

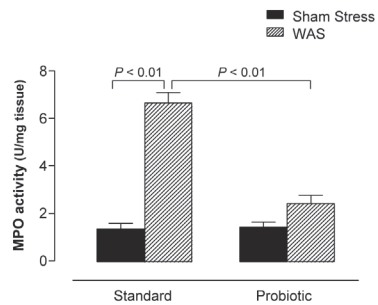


FIGURE 6

Probiotics decrease stress-induced mucosal RMCP-II levels.

After seven days of standard diet (n=8) or probiotics (n=8) wild type +/+ rats were subjected to water avoidance stress (WAS) (n=8) or sham stress (n=8) for 10 consecutive days. Rat mast cell proteinase (RMCP)–II was determined in colonic mucosa. Graph shows mean ± SEM. Comparisons were preformed using ANOVA followed by Tukey's HSD.

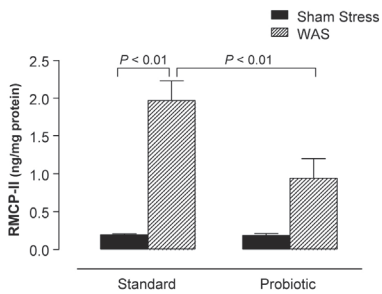


FIGURE 7

Probiotic protective effects are mast cell dependent

Ws/Ws, mast cell deficient rats were treated with a standard diet (n=8) or probiotics (n=8) and subjected to water avoidance (WAS) stress (n=8) or sham stress (n=8) for 10 consecutive days. Colonic mucosa was mounted in Ussing chambers. In mast cell deficient rats probiotics did not have an effect on stress-induced (A) increase in base line conductance, (B) enhanced ^{51}Cr-EDTA flux, or (C) passage of live fluorescent *E. coli* HB 101. (D) In addition, probiotics did not prevent stress-induced mucosal inflammation as measured by myeloperoxidase (MPO) activity. Graphs show means ± SEM. Data were collected from independently acquired sets of three tissue segments per rat. Comparisons were preformed using ANOVA followed by Tukey's HSD.

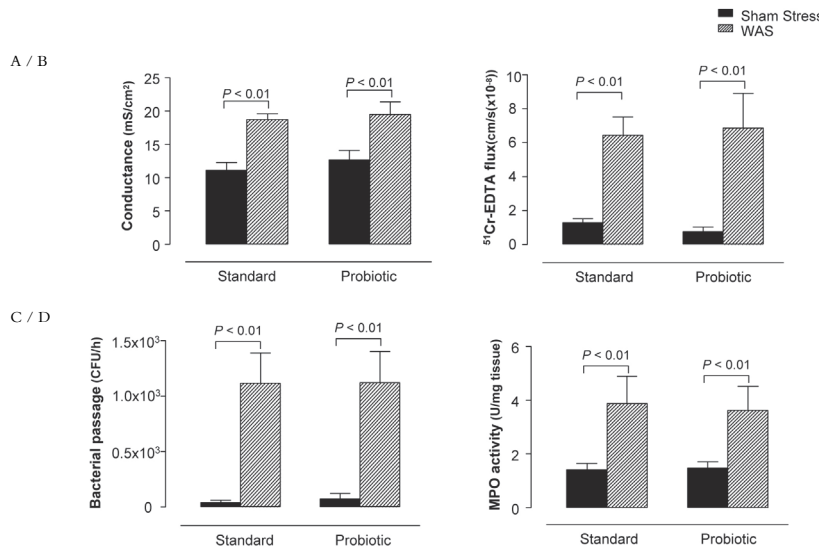


FIGURE 8

Analysis of colonic PPAR-γ activation

32 Mast cell deficient (Ws/Ws) and 32 wild-type (+/+) rats were subjected to water avoidance stress (WAS) or sham stress (SS) 1hr/day for 10 days. Seven days prior to the onset of stress, probiotics were added to the standard diet in half of the animals. (A) PPAR-γ transcription factor in nuclear fractions, and (B) 15d-PGJ_2 levels were determined in colonic mucosa. Graphs show means ± SEM. Analyses were run in duplicates. Comparisons were preformed using ANOVA followed by Tukey's HSD.

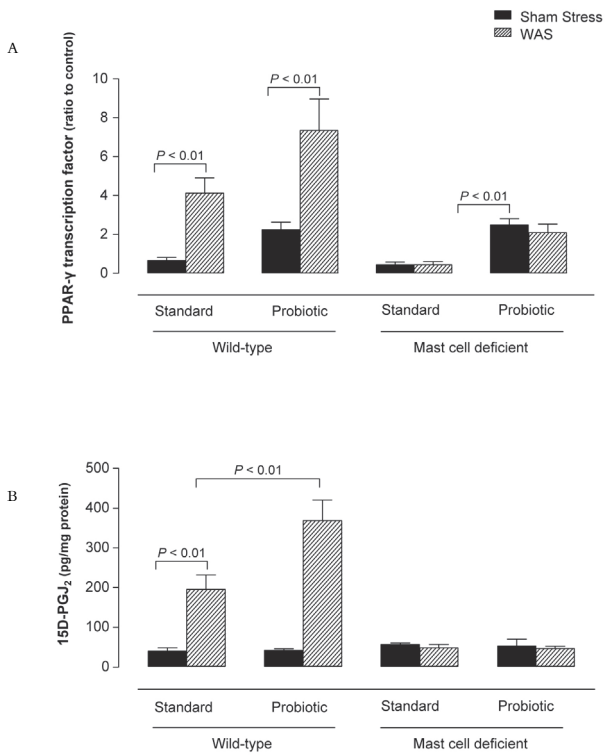
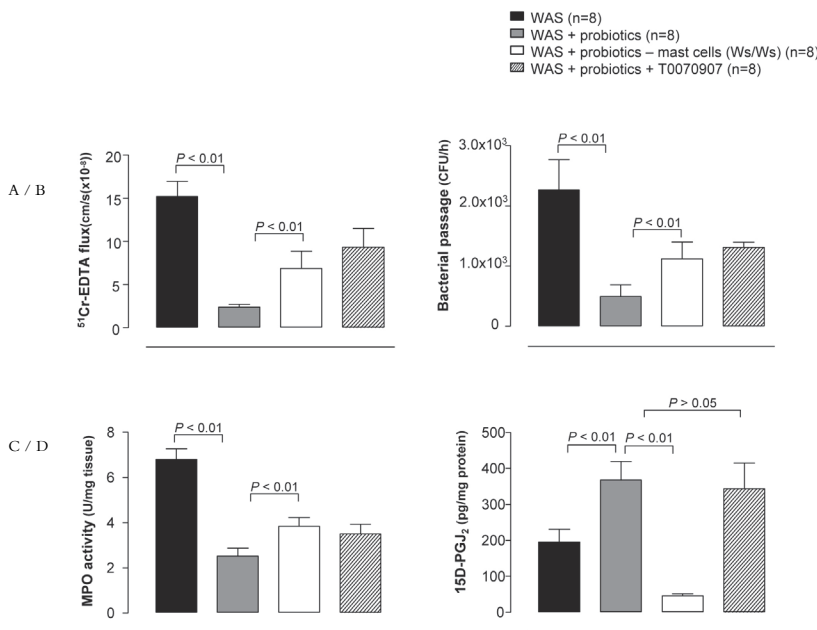


FIGURE 9

Probiotic-induced barrier protective effects are mast cell and PPAR-γ dependent

8 Mast cell deficient and 16 wild-type rats were subjected to water avoidance stress (WAS) or sham stress (SS) 1hr/day for 10 days. Seven days prior to the onset of stress, probiotics were added to the standard diet in all mast cell deficient and half of the wild-type animals. To determine dependence of PPAR-γ an additional 8 WAS subjected, probiotic-fed wild-type rats were injected daily with the specific PPAR-γ antagonist T0070907. Colonic mucosa was mounted in Ussing chambers to determine (A) ^{51}Cr-EDTA flux, and (B) passage of live fluorescent *E. coli* HB 101. (C) Myeloperoxidase (MPO) activity was quantified in colonic mucosa to determine mucosal inflammation. (D) Mucosal 15d-PGJ_2 levels in colonic mucosa. Graphs show means ± SEM. Data were collected from independently acquired sets of three tissue segments per rat. Comparisons were preformed using ANOVA followed by Tukey's HSD.



DISCUSSION

In this study, we expanded on previous findings that probiotics effectively maintain intestinal barrier integrity and ameliorate mucosal inflammation in a rat model of chronic stress[41]. Oral administration of probiotic bacteria resulted in decreased levels of RMCP-II, enhanced mucosal 15d-PGJ_2 levels and elevated PPAR-γ activation. Furthermore, both in mast cells deficient animals and after inhibition of PPAR-γ, the ability of probiotic bacteria to maintain intestinal barrier function was abrogated. These novel data strongly suggest that mast cells contribute to the protective effects of probiotics in stress-induced mucosal dysfunction.

Stress is a common experience in daily life and the influence of stress on outcome of gastrointestinal disorders is well established[42]. Stressful life events worsen the clinical course of Crohn's disease and prolonged stress increases the number of relapsed in ulcerative colitis patients[43]. Chronic WAS in rats is a widely used model of chronic psychological stress and utilizing this model, investigators have shown that this chronic form of stress causes prolonged intestinal barrier dysfunction[44]. This increase in mucosal permeability allows for antigens and bacteria to penetrate the intestinal barrier, causing mucosal inflammation. Psychological stress is indeed associated with clinical symptoms and outcome of chronic intestinal disorders[45]. Our data are consistent with these findings: ten days of chronic stress impaired the intestinal barrier function via transcellular and paracellular routes, increased colonic permeability to bacteria and induced bacterial translocation to MLNs. Treatment with probiotics ameliorated both mucosal barrier dysfunction and bacterial translocation in response to stress.

Bacterial adhesion of potential pathogens plays a pivotal role in mucosal infections and bacterial translocation[46]. The current study shows that stress-induced bacterial adhesion was decreased after probiotic pretreatment. Interestingly, administration of probiotics prevented the stress-induced depletion of *Lactobacillus* species that associated with the epithelium, which is believed to be essential to their mechanism of action[47].

The reduction in colonic barrier dysfunction after exposure to chronic stress was associated with a probiotic-induced reduction in MPO activity, indicative of an amelioration of mucosal inflammation[48]. These findings are in agreement with previous studies showing that administration of probiotics attenuates mucosal barrier dysfunction and diminishes bacterial translocation in different models of psychological stress[49]. Limiting mucosal permeability may be a crucial factor in amelioration of stress-induced clinical symptoms as invasion of antigens and bacteria initiates and maintains mucosal inflammation, which will only exacerbate the mucosal barrier failure[50].

41 Zareie *et al.*, 2006
42 Lutgendorff *et al.*, 2008
43 Levenstein *et al.*, 2000
44 Soderholm *et al.*, 2002;Velin *et al.*, 2004
45 Wilhelmsen, 2000
46 Soderholm *et al.*, 2002
47 Lutgendorff *et al.*, 2008
48 Smith & Castro, 1978
49 Gareau *et al.*, 2007; Zareie *et al.*, 2006
50 Soderholm & Perdue, 2006

Stress is known to affect mucosal barrier integrity by routes involving mast cells[51]. Upon activation mast cells release a large variety of pro-inflammatory mediators, e.g. histamine and TNF-α, which negatively affects mucosal barrier function[52]. Previous studies have shown that the production of these mediators by mast cells can be modulated by other factors found at inflammatory sites, such as IFN-γ[53], PGE_2[54] and IL-10[55]. Furthermore, certain commensal microbes are suggested to inhibit mast cell degranulation[56]. In our study stress-induced elevation of RMCP-II was reduced in animals treated with probiotics, suggesting that probiotic treatment inhibited and or modulated degranulation patterns of mast cells. This may have been due to a primary effect of probiotics on mast cells or alternatively through an upregulation of IL-10, as it is known to be induced by probiotic bacteria[57]. Intriguingly, in the present study we were able to show that the protective effects of probiotics on gut barrier function and mucosal inflammation appeared to be dependent on the presence of mast cells, as probiotic treatment of stress exposed Ws/Ws rats did not yield a significant amelioration of mucosal barrier dysfunction nor did it alleviate mucosal inflammation.

The ability of probiotics to maintain mucosal barrier function during chronic subjection to stress, may involve a probiotic-induced inhibition of inflammation through effects on PPAR-γ[58]. While a wide range of cell types including macrophages, neutrophils, fibroblasts and dendritic cells, are known to synthesize various forms of prostaglandins, mast cells produce mainly PGD_2[59]. 15d-PGJ_2 is derived from PGD_2 and is an endogenous, highly selective agonist for PPAR-γ. Here we provide novel *in vivo* evidence that stress-induced elevation in mucosal 15d-PGJ_2 is mast cell dependent and that oral feeding of rats with probiotics further enhances the levels of 15d-PGJ_2 after exposure to stress. The stress induced increase in 15d-PGJ_2 seems to run parallel with the activation of PPAR-γ. However the fact that PPAR-γ activity was increased after probiotic treatment of sham stressed and mast cell deficient animals, indicates that probiotic-induced PPAR-γ activation was not solely dependent on 15d-PGJ_2 induction. This may be explained by previous work showing production of conjugated linoleic acid by probiotics, which is known to be a PPAR-γ ligand[60]. Also, the degradation of PPAR-γ occurs through the ubiquitin-proteasome system[61] and probiotics have previously been shown to reduce cellular proteasomal activity[62]. It is therefore possible that probiotics decrease the degradation of PPAR-γ and thereby maintain its activity.

PPAR-γ appears to be involved in mediating the probiotic-induced protection of mucosal barrier function and amelioration of mucosal inflammation, in that the PPAR-γ antagonist T0070907 abolished the protective effects of probiotics after exposure to stress. Both endogenous and pharmaceutical ligands of PPAR-γ

51 Santos *et al.*, 1999
52 Soderholm *et al.*, 2002
53 Bissonnette *et al.*, 1995
54 Leal-Berumen *et al.*, 1995
55 Marshall *et al.*, 1996
56 Magerl *et al.*, 2008
57 Timmerman *et al.*, 2007
58 Madsen *et al.*, 2001
59 Lewis *et al.*, 1982
60 Ewaschuk *et al.*, 2006
61 Clark, 2002
62 Jijon *et al.*, 2004; Petrof *et al.*, 2004

STRESS IS A COMMON EXPERIENCE IN DAILY LIFE

PRETREATMENT WITH PROBIOTICS IS EFFECTIVE IN PROTECTING THE GUT AGAINST STRESS-INDUCED INTESTINAL BARRIER DYSFUNCTION AND MUCOSAL INFLAMMATION

have been shown to be efficacious in reducing stress-induced barrier dysfunction[63]. Nonetheless, this is the first study to show PPAR-γ mediated probiotic effects on barrier function in a model of chronic stress. Based on the current data however, it is impossible to distinguish whether the protective effects afforded by probiotics were entirely dependent on PPAR-γ activated by 15d-PGJ_2, derived from mast cells or that the protective effects were also mediated through a direct probiotic-induced activation of PPAR-γ within epithelial cells. More mechanistically directed experiments are warranted to determine if possible direct effects of probiotics on mucosal PPAR-γ are also contributing factors to their barrier protective properties.

In conclusion, pretreatment with these multispecies probiotics is effective in protecting the gut against stress-induced intestinal barrier dysfunction and mucosal inflammation. Furthermore, this protection involves a mast cell and PPAR-γ depended mechanism, which is putatively mediated through mast cell-derived 15d-PGJ_2. This novel mechanism of action of probiotics sheds more light on their anti-inflammatory properties and represents a potential adjunctive therapeutic option for the prevention of stress-induced symptoms and the reduction in the number of relapses in IBD.

63 Ponferrada *et al.*, 2007

10

Probiotics modulate mast cell degranulation and reduce stress-induced barrier dysfunction *in vitro*.

Probiotics change mast cell release

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ABSTRACT

BACKGROUND

Stress has well-established deleterious effects on intestinal barrier function and stressful life events are known to contribute to the development and perpetuation of inflammatory bowel diseases. Mast cells play a pivotal role in pathogenesis of stress-induced barrier dysfunction due to the release of barrier disruptive content. Conversely, they also have recently been suggested to contribute to barrier protective properties of probiotics, through the release of 15d-PGJ_2 and enhanced epithelial PPAR-γ activity. However, mechanisms remain to be elucidated. The aim was to study if probiotics can modulate mast cell mediator release, resulting in amelioration of stress-induced barrier dysfunction *in vitro.*

METHODS

Confluent monolayers of the human colon-derived T84 epithelial cell line were co-cultured with rat basophilic leukemia (RBL)-2H3 mast cells and pretreated with probiotics (Ecologic® 825)[1] before addition of CRH[2] to activate mast cells. Release of beta hexosaminidase, TNF-α and 15d-PGJ_2 from mast cells was determined. Transepithelial resistance (TER), and permeability to microspheres (0.2 μm) were measured over a 24h period. To determine dependence of PPAR-γ, monolayers were incubated with the specific PPAR-γ antagonist T0070907 before treatment with probiotics.

1 $125x10^4$ CFU/ml, 1hr
2 100nM CRH

RESULTS

CRH-induced activation of mast cells resulted in decreased TERs and increased permeability to microspheres. Both pretreatment with probiotics and filter-sterilized probiotic supernatant resulted in lower levels of mast cell-released beta hexosaminidase and TNF-α and increased 15d-PGJ_2. Furthermore, probiotics ameliorated epithelial barrier dysfunction in monolayers exposed to CRH-activated mast cells. However, when T84 monolayers were exposed to conditioned medium of CRH-activated mast cells or were incubated with T0070907, probiotics showed little or no effect.

CONCLUSIONS

Probiotics modulate mast cell mediator profiles to release a more barrier protective profile, resulting in amelioration of stress-induced epithelial barrier dysfunction, which is putatively mediated by a PPAR-γ dependent pathway.

INTRODUCTION

Psychological stress has been recognized as factor in exacerbation and relapse of inflammatory bowel disease[3] and functional bowel diseases, probably by disrupting the intestinal barrier function by routes involving mast cells[4]. As adequate responses to these stressors are necessary for survival, evolutionary pressure resulted in sophisticated defense mechanisms to counteract the negative effects of stress and other threats to homeostasis. One of such defense mechanisms that has been associated with chronic stress is increased activation of peroxisome proliferator-activated receptor (PPAR)-γ[5]. PPARs are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes. One of the isoforms, PPAR-γ, is known for its anti-inflammatory effects and has been shown to be upregulated during chronic stress, affording intestinal barrier protective effects[6]. Activation of PPAR-γ occurs through 15-deoxy-Δ prostaglandin J_2 (15d-PGJ_2)[7], a prostaglandin (PG) D_2 derivative of which enhanced intestinal levels has been shown during stress[8]. Furthermore, PPAR-γ activation ameliorates intestinal inflammation in several models of experimental colitis[9].

Mast cells may have a dual role during stress. There is extensive evidence that upon stress, mast cells release factors e.g. TNF-α and histamine, that disrupt the mucosal barrier[10] and previous work showed that the deleterious intestinal effects evoked by stress are mast cell dependent[11]. Conversely, mast cells are also the primary source of 15d-PGJ_2 the endogenous, high affinity agonist of PPAR-γ[12]. By providing a means of PPAR-γ activation, mast cells may play a key role in this anti-inflammatory defense mechanism. Indeed as our group previously showed stress-induced PPAR-γ activation is significantly reduced in mast cell deficient rats[13].

Commensal intestinal microbiota play a pivotal role in intestinal homeostasis and mucosal barrier function[14]. Probiotics have been shown to ameliorate the deleterious effects of stress on intestinal function, but mechanisms remain to be elucidated[15]. In a rat model of chronic stress our group has recently shown that these barrier protective effects of probiotics are dependent of mast cells and PPAR-γ activation[16]. In addition, commensal bacteria, as for instance non-pathogenic *Escherichia coli*, can modulate degranulation of mast cells[17].

Given the dual role of mast cells and keeping in mind that mast cells contain compounds that can either harm (e.g. TNF-α) or protect (e.g. 15d-PGJ_2) the intestinal barrier, we hypothesized that pretreatment with probiotics may modulate the

3 Levenstein *et al.*, 2000
4 Soderholm *et al.*, 2002; Santos *et al.*, 2008
5 Ponferrada *et al.*, 2007
6 Ponferrada *et al.*, 2007
7 Kliewer *et al.*, 2001
8 Lutgendorff *et al.*, Chapter 9
9 Su *et al.*, 1999; Desreumaux *et al.*, 2001
10 Santos *et al.*, 2001
11 Soderholm *et al.*, 2002
12 Kobayashi *et al.*, 2005
13 Lutgendorff *et al.*, Chapter 9
14 Madsen *et al.*, 2001; Garcia-Lafuente *et al.*, 2001
15 Gareau *et al.*, 2007; Eutamene *et al.*, 2007
16 Lutgendorff *et al.*, Chapter 9
17 Magerl *et al.*, 2008

EVOLUTIONARY PRESSURE RESULTED IN SOPHISTICATED DEFENSE MECHANISMS TO COUNTERACT NEGATIVE EFFECTS OF STRESS AND OTHER THREATS TO HOMEOSTASIS

MAST CELLS MAY CHANGE THEIR ROLE FROM BEING PRIMARILY DELETERIOUS TO HAVING PROTECTIVE EFFECTS DURING CHRONIC STRESS

function of mast cells. We found a change in mediator release profile with increased release of 15d-PGJ_2 and lower levels of pro-inflammatory mediators. Due to this shift in mediator release towards a more intestinal barrier protective profile, mast cells, under the influence of probiotics, may change their role from being primarily deleterious to having protective effects during chronic stress.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

The human colon-derived T84 epithelial cell line was cultured at 37°C with 5% CO2 in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 2% (v/v) penicillin-streptomycin and 1.5% (v/v) HEPES (all from Invitrogen, Burlington, Burlington,ON, Canada) and 10% fetal bovine serum (CanSera, Toronto, ON, Canada). T84 cells (passages 70-90) were seeded at a density of 10^6 cells/ml onto semipermeable filters with 3 μm pore size and a surface area of 1.1 cm^2 (Costar, Cambridge, MA) until transepithelial electrical resistance (TER) was 900-3000 W/cm^2 (typically 7–10 days, medium changed every 1–2 days). TER (as an accepted index of paracellular permeability[18]) of each monolayer was measured before and 24 hr after treatment and is expressed as the percentage of pretreatment TER values to normalize for variation in absolute values between individual monolayers[19]. To further characterize epithelial barrier function, permeability to fluorescent microspheres (0.2 μm, FluoSpheres, Invitrogen, Carlsbad, CA, USA) was analyzed. FluoSpheres® (2.2 x10^9 beads/ml) were added to the apical side of the T84 monolayers. Samples (50μl) were collected from the basolateral compartment 24 hr later and concentration of translocated beads was determined by fluorescent measurement at 515nm, using a standard curve of known concentrations. Results are expressed as percentage of apically added FluoSpheres®, recovered in the basal chambers over 24 hrs.

The rat mast cell line RBL-2H3 (ATCC CRL-2256) were maintained as monolayer culture in 75 cm^2 tissue flasks in Eagles minimum essential media supplemented with 10% of heat inactivated fetal calf serum and 100 units/ml penicillin and 100 μg/ml streptomycin and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For secretion studies, cells were dislodged from the flasks with trypsin (0.05%) and EDTA (0.02%) solution, resuspended in and rinsed in media then plated in 24-well plates at 2.5 × 10^5 cells/well for 3hrs. Mast cell conditioned media was obtained by incubating mast cells for 30 min with CRH (100nM) at 37°C after which supernatant was collected.

Probiotics (*Ecologic® 825*, Winclove Bio Industries, Amsterdam, The Netherlands) consisted of nine viable and freeze-dried probiotic strains; *Bifidobacterium bifidum* (W23), *Bifidobacterium lactis* (W52), *Bifidobacterium lactis* (W51), *Lactobacillus acidophilus* (W22), *Lactobacillus casei* (W56), *Lactobacillus paracasei* (W20), *Lactobacillus plantarum* (W61), *Lactobacillus salivarius* (W24), *Lactococcus lactis* (W19). The probiotic mixture was rehydrated to the indicated concentration in antibiotic free-medium.

RBL mast cells or T84 monolayers were exposed to 25x10^4, 12.5x10^5, 25x10^5, 12.5x10^6, or 25x10^6 CFU/ml probiotics for 1 hr before stimulation with the indicated dose of CRH (Sigma, Oakville, ON Canada) or 5μM A23187 (Sigma). Cells were also incubated with probiotic conditioned medium, which was obtained by passing probiotic solutions (25x10^4, 12.5x10^5, 25x10^5, or 12.5x10^6, CFU/ml) though

18 McKay *et al.*, 2007
19 Watson *et al.*, 2004

a 0.45 μm sterile filter after 1 hr of incubation at 37°C. The obtained filter sterilized probiotic medium was used immediately to pre-treat mast cells or T84 monolayers.

Furthermore, mast cells were pretreated with probiotics killed by paraformaldehyde (PFA). These were obtained by incubating 1 ml of probiotic solutions ($25x10^4$, $12.5x10^5$, $25x10^5$, or $12.5x10^6$, CFU/ml) with 9 ml of 4% PFA at 37°C for 10 min. PFA was then removed by 4x washing with PBS and the PFA-killed probiotics were added to antibiotic-free medium, before the start of pretreatment.

In addition, mast cells were exposed for 1 hr to *Escherichia coli* HB 101 ($1x10^4$, $5x10^4$, $1x10^5$, $5x10^5$, or $1x10^6$ CFU/ml). Bacteria were grown and cultured over night in an orbital shaker in Luria-Bertani (LB) broth at 37°C before addition to the mast cells.

BETA HEXOSAMINIDASE, TNF-α AND 15-DEOXY-Δ PROSTAGLANDIN J_2 RELEASE ASSAY

Mast cells were incubated for 30 min with 100nM CRH or 5μM A23187 as a positive control and ELISA for TNF-α (R&D Systems, Minneapolis, MN, USA) and 15-deoxy-Δ prostaglandin J_2 (Assay Designs, Ann Arbor. MI, USA) was performed according to manufacturer's instructions. Beta hexosaminidase release was analyzed in a 48-well plate, 2.5 x 10^5 cells/well. After mast cell stimulation, 50 μl of the supernatant was transferred and incubated with 200μl of 1mM p-nitrophenyl N-acetyl-beta-D-glucosamine in 0.05M citrate buffer (pH 4.5) for 1hr at 37°C. As a control for total beta-hexosaminidase concentration the cells were lysed with 1% Triton X-100 and 50μl of the cytosolic fraction was transferred and incubated similar to normal supernatant. The reactions were quenched by addition of 500μl of 0.05M sodium carbonate buffer (pH 10.0). The optical density was read at 405nm using Perkin Elmer Victor 3 multi label plate reader (PerkinElmer, Waltham, MA, USA). Data are expressed as percentage released beta hexosaminidase of total.

MAST CELL VIABILITY

Cell viability was evaluated by measuring the mitochondrial-dependent reduction of colorless 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a colored blue formazan. RBLs were seeded in a 96-well plate at a density of 0.1 $x10^6$ cells/well. After reaching confluency, cells were exposed for 1 hr to probiotics ($25x10^4$, $12.5x10^5$, $25x10^5$, $12.5x10^6$ and $25x10^6$ CFU). After the treatment period, experimental treatments were aspirated from each well and cells were washed with phenol-free RPMI medium, supplemented with 10%FBS and 36μM HEPES. Following 4 hrs of incubation with 100μl MTT (0.5mg/ml) at 37°C, the medium was removed and replaced by 50 μl acidic isopropanol (0.04N HCL in absolute isopropanol). Absorbance was measured at 595nm.

CO-CULTURE OF MAST CELLS AND EPITHELIAL CELLS

In co-culture experiments RBL-2H3 mast cells were seeded at a concentration of $2.5x10^5$ cells/well in the bottom of 12-well plates on the day of the experiment. After three hours of incubation the media was changed to T84 media and Transwell

filters with confluent T84 monolayers were transferred to the wells. The cells were left to acclimatize for one hour and then 100nM CRH were added to the basolateral chamber to activate the mast cells. TER was measured after 24 hours. In separate experiments filter-grown T84 monolayers were challenged by exposure to mast cell-conditioned media. To obtain conditioned media, mast cells were exposed to 100nM CRH for 30 minutes and supernatant was transferred to wells with Transwell filters.

PPAR-γ ACTIVATION

Plastic grown T84 monolayers were treated with 1) conditioned medium of mast cells that were either pretreated or not with probiotics ($125x10^4$ CFU/ml, 1hr) or 2) were incubated with probiotics ($125x10^4$ CFU/ml) or 3) their filter sterilized supernatant for 1 hr. To determine activated PPAR-γ, nuclear protein extracts were obtained, and protein concentrations were determined using the Bradford assay[20]. PPAR-γ transcription factor levels were quantified using a PPAR-γ transcription factor ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) as indicated by the manufacturer. Results were normalized to nuclear protein and are expressed as ratio to controls.

STATISTICAL ANALYSIS

Data are presented as mean ± standard error of the mean (SEM), where n values indicate the number of individual epithelial monolayers or confluent mast cell cultures from a specified number of experiments. All data were assessed for normal distribution using Shapiro–Wilk's test. Parametric values are presented as mean (SEM). Statistical analysis was performed by ANOVA followed by Tukey's HSD test for multiple comparisons (SPSS 13.0 Inc, Chicago, Illinois, USA). *P* values <0.05 were considered significant.

20 Bradford, 1976

RESULTS

PROBIOTICS REDUCE CRH-INDUCED DEGRANULATION AND TNF-α RELEASE

Initial experiments examined the ability of CRH to cause degranulation of RBLs. Stimulation of mast cells with CRH caused a dose-dependent and time-dependent release of hexosaminidase from RBLs (fig 1). After 30 minutes this increase in hexosaminidase release was significant ($p<0.05$) at a concentration of CRH of 10nM, but was more substantial at a concentration of 100nM. Mast cells treated with calcium ionophore A23187 (5μM), as a positive control for mast cell activation, secreted similar levels of hexosaminidase, 30 minutes after activation as compared to CRH (100nM) treated mast cells. Pretreatment with probiotics inhibited CRH-induced degranulation in a dose dependent manner over the range $25x10^4$ – $25x10^6$ CFU/ml, with maximal effect at $25x10^5$ CFU/ml (fig 2 A). A concurrent decrease in CRH-induced TNF-α release was observed in probiotic pretreated mast cells (fig 2 B). This reduction in TNF-α release coincided with an increased release of 15d-PGJ_2 after CRH stimulation (fig 2C). No significant release of TNF-α or 15d-PGJ_2 was observed following probiotic treatment in the absence of CRH or A23187.

CRH or A23187-induced activation of mast cells was not prevented by incubation with *E. coli* HB101, a non-pathogenic laboratory strain (fig 3). Of note, incubation of mast cells with probiotics, without addition of a mast cell activating agent, did not induce significant spontaneous degranulation (fig 2A) and did not affect viability of mast cells (data not shown).

Taken together the above, probiotics reduce both CRH- and A23187-induced degranulation of RBLs dose-dependently, contrary to the commensal *E. coli* HB101 strain. In addition pretreatment with probiotics modulates the released mediator profile by reducing TNF-α release and increasing the production of 15d-PGJ_2.

INHIBITORY EFFECTS OF PROBIOTICS ARE STRAIN SPECIFIC AND PUTATIVELY MEDIATED THROUGH SOLUBLE FACTORS

To test whether the found effects were dependent on the viability of the probiotics, mast cells were pretreated with PFA-killed probiotics, which had no inhibitory effect on CHR-induced mast cell degranulation (fig 4A). To further characterize the mechanism by which probiotics modulate CRH-induced mast cell degranulation, mast cells were pretreated with filter-sterilized probiotic supernatant, to determine whether soluble factors produced by probiotics were able to sort effect. Compared with medium control, filter-sterilized probiotic-supernatant decreased CRH-induced degranulation of mast cells in a dose dependent manner (fig 4B). Furthermore, both TNF-α and 15d-PGJ_2 release were affected by pretreatment with probiotic supernatant. While pretreatment with supernatant increased 15d-PGJ_2 release, TNF- α levels in media of mast cells activated with CRH, was decreased. This suggests that soluble factors released by viable probiotics rather than surface factors on the probiotic strains mediate the inhibitory effects on mast cell degranulation and modulation of mediator profile.

To determine strain specificity, mast cells were pretreated with $25x10^5$ CFU/ml of the different strains of which the probiotic mixture is composed. No general effects of either lactobacilli or bifidobacteria strains were detected. *L. paracasei,* (W20), *B. bifidum* (W23), *L. salivarius* (W24), *B. infantis* (W52), and *L. casei* (W56) significantly reduced CRH-induced release of beta hexosaminidase, whereas the other strains showed no inhibitory effects (fig 6).

CRH-INDUCED ACTIVATION OF MAST CELLS DISRUPTS BARRIER FUNCTION

Mast cells are known to be involved in stress-induced mucosal barrier dysfunction[21] and it is assumed that upon activation mast cells release their barrier disruptive content. For this reason mast cells were co-cultured in the basolateral chamber of a transwell system; TER and passage of fluorescent microspheres (0.2 μm) were chosen as indicators of barrier function of T84 monolayers. Fluorescent microspheres are biologically inert beads of which only small amount are transported to the subepithelial region under physiological conditions. The results showed that <0.05% of the original microspheres added to the apical chambers were detected in samples from the basolateral side in naïve control T84 layers (fig 7B). Activation of mast cells by addition of CRH (100nM) caused marked decreases in TER and enhanced the permeability to microspheres (fig 7 A, B). Of note, addition of CRH to the basolateral side of the trans-well system in absence of mast cells did not affect barrier function of T84 monolayers (fig 7 A, B).

PROBIOTICS MODULATE MAST CELL MEDIATOR RELEASE AND PROTECT BARRIER FUNCTION

To evaluate whether probiotics added to the apical side of T84 cells, affect mediator release of mast cells, co-cultures of mast cells with filter grown T84 cells were pretreated with probiotics ($25x10^4$-$25x10^5$ CFU/ml) 1hr before addition of CRH (100nM) to the basal lateral side. Despite being separated from mast cells by the T84 monolayer, pretreatment with probiotics decreased the release of beta hexosaminidase and TNF-α and increased levels of 15d-PGJ_2 in supernatant of basal lateral side of the transwell (fig 8 A, B, C). This change in mediator release coincided with a protective effect of probiotics on deleterious effects of mast cells on barrier function. This was reflected in an amelioration of the drop in TER after activation of the mast cells and maintenance of the barrier function against microspheres (fig 8 D, E). Of note, treatment of filter-grown T84 cells with probiotics did not lead to an increase in TER, compared to untreated confluent monolayers of T84 cells (data not shown).

BARRIER PROTECTIVE EFFECT OF PROBIOTICS IS PARTIALLY MEDIATED THROUGH MAST CELLS

We next investigated whether barrier protective effects of probiotics were dependent on their interaction with mast cells. Exposure of T84 monolayers to

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supernatant from CRH-activated mast cells caused a marked decrease in TER, similar to reduction in TER after activation of mast cells in co-culture with T84 cells. In spite of the absence of mast cells, pretreatment with probiotics ameliorated the barrier disruptive effects of supernatant from CRH-activated mast cells, although not to the extent of the protective effects exerted by probiotics in the presence of mast cells (fig 9 A, B). This suggests that mast cells contribute to the barrier protective effects of probiotics.

MAST CELL-MODULATING PROPERTIES OF PROBIOTICS ARE NOT DEPENDENT ON BACTERIAL TRANSLOCATION

To assess whether barrier protective effects of probiotics were dependent on bacterial translocation of the probiotics, T84 filter-grown monolayers were pretreated with filter sterilized probiotic supernatant. Upon activation of mast cells with CRH (100nM), filter sterilized probiotic supernatant demonstrated similar barrier protective effects as compared to pretreatment with probiotics (fig 10 A, B). Assessment of supernatant of the basolateral side of the transwell system demonstrated that also probiotic supernatant was able to modulate mast cell mediator release (fig 10 C, D, E). This may have been due to probiotic soluble factors penetrating the epithelial monolayer and modulating mast cell properties. Alternatively probiotics and or their soluble factors could act via epithelial cells to trigger the release of mast cell modulating factors from epithelial cells.

To test if these barrier protective effects were in a similar fashion to probiotic pretreatment, dependent on modulation of mast cells, T84 monolayers were after pretreatment with probiotic supernatant exposed to supernatant from CRH-activated mast cells. Interestingly, pretreatment of T84 monolayers in the absence of mast cells had no protective effects on barrier disruption induced by supernatant from CRH-activated mast cells (fig 11 A, B).

BARRIER PROTECTIVE EFFECTS OF PROBIOTICS ARE DEPENDENT ON PPAR-γ ACTIVATION.

PPAR-γ has been shown to exert anti-inflammatory and barrier protective effects *in vivo* and can be activated by its endogenous agonist 15d-PGJ_2[22]. To test the hypothesis that the increased release of 15d-PGJ_2 by probiotic-treated mast cells increased PPAR-γ, plastic-grown T84 cells were treated with conditioned medium of activated mast cells, treated with or without probiotics. Conditioned medium of probiotic-pretreated, CRH-activated mast cells caused a marked increase in PPAR-γ activation in T84 cells as compared to supernatant of activated mast cells that were not pretreated with probiotics (fig 12A). Furthermore, nuclear PPAR-γ transcription factor was also increased in T84 cells after treatment with probiotics. However, treatment with probiotic supernatant did not yield any effects on PPAR-γ activation (fig 12A).

To determine whether barrier protective effects of probiotics were dependent or independent on PPAR-γ activation, T84 monolayers were prepared with T0070907

22 Ponferrada *et al.*, 2007

(1μM), a selective PPAR-γ antagonist, before pretreatment with probiotics. The protective barrier effects of probiotics or their supernatant could not be reproduced in T0070907 treated monolayers. Mast cell activation-induced dysfunction of the epithelial barrier in T0070907 + probiotic treated monolayers was similar to barrier dysfunction in untreated monolayers exposed to activated mast cells (fig 12B, C).

FIGURE 1

CRH induces degranulation of mast cells in a dose dependent fashion

Figure showing that incubation of RBL mast cells with CRH at varying dosages (5μM, 1 μM, 100nM, 10nM) causes a dose dependent degranulation of mast cells as gauged by the release of beta hexosaminidase. Data are mean ± SEM; n=6 preparations from 2 experiments; A23187 was included as a positive control (5 μM). Comparisons were preformed using ANOVA followed by Tukey's HSD

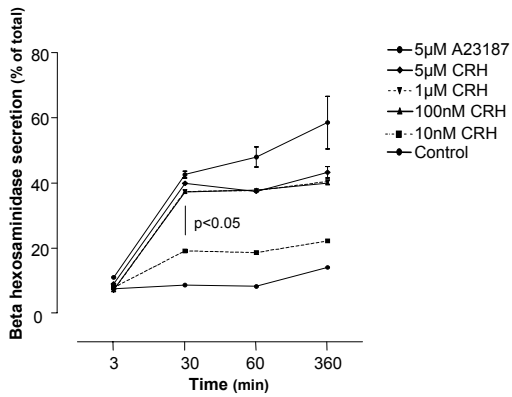


FIGURE 2

Pretreatment with probiotics modulates CRH-induced mediator release from mast cells

(A) Bar graph showing that 1hr pretreatment with probiotics does not induces spontaneous degranulation and decreased both CRH- and A23187-induced release of beta hexosaminidase in a dose dependent fashion. (B) Bar graph showing that 1hr pretreatment with probiotics reduced both CRH- and A23187-induced release of TNF-α dependent of the used probiotic dose. (C) Bar graph showing that 1hr pretreatment with probiotics results in increased CRH- and A23187-induced release of 15d-PGJ_2 Data are mean ± SEM; n=6 preparations from 2 experiments. Comparisons were preformed using ANOVA followed by Tukey's HSD.

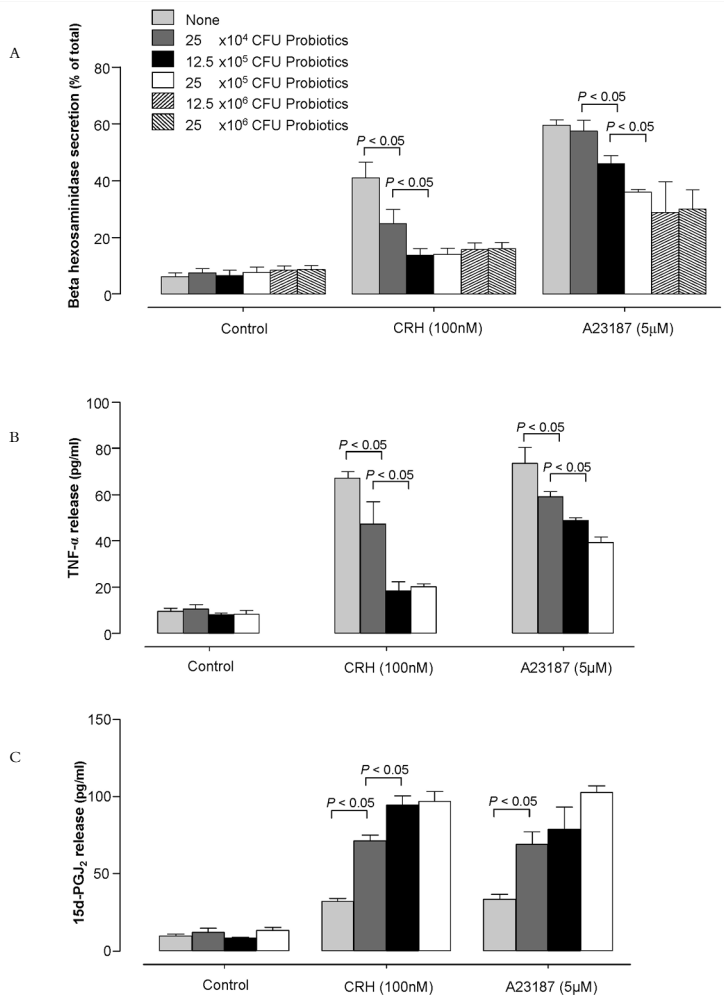


FIGURE 3

Exposure to *Escherichia coli* HB101 does not affect mast cell degranulation

RBL mast cells were exposed to varying concentrations of *E. coli* HB101 for 1 hr and subsequently activated by addition of 100nM CRH or 5μM A23187. Data are mean ± SEM; n=6 preparations from 2 experiments. Comparisons were preformed using ANOVA followed by Tukey's HSD.

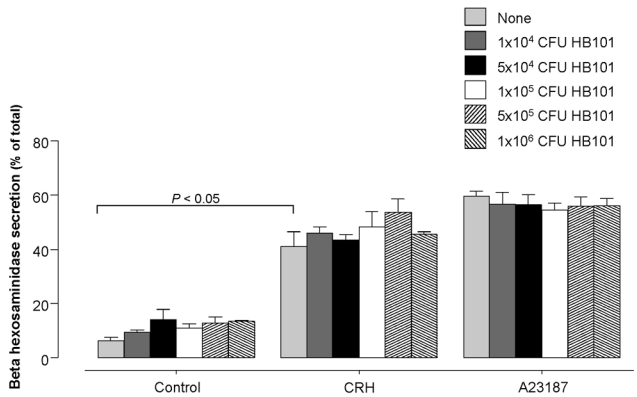


FIGURE 4

Probiotic soluble factors affect mast cell degranulation

(A) Bar graph showing that 1hr pretreatment with varying dosages of PFA-killed probiotics did not affect CRH- or A23187-induced beta hexosaminidase release. (B) Probiotics, at the indicated dose, were incubated with medium for 1hr at 37°C. Filter-sterilized supernatant was used to pretreat RBL mast cells for 1hr after which CRH (100nM) as added to the culture. Data are mean ± SEM; n=6 preparations from 2 experiments. Comparisons were preformed using ANOVA followed by Tukey's HSD.

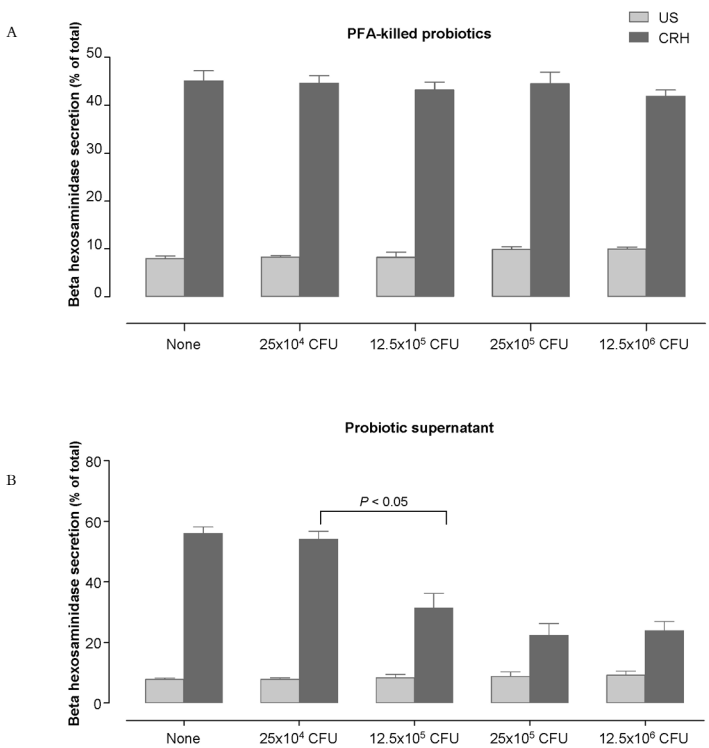
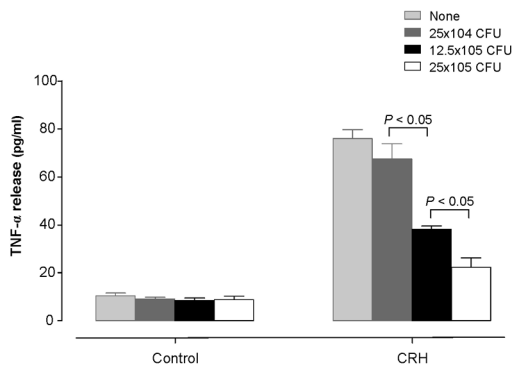


FIGURE 5

Probiotic soluble factors modulate mast cell mediator release

Probiotics, at the indicated dose, were incubated with medium for 1hr at 37°C. Filter-sterilized supernatant was used to pre-treat RBL mast cells for 1hr after which CRH (100nM) as added to the culture. (A) Release of TNF-α into the supernatant as measured by ELISA. (B) Release of 15d-PGJ_2 into the supernatant. Data are mean ± SEM; n=6 preparations from 2 experiments. Comparisons were preformed using ANOVA followed by Tukey's HSD.

A



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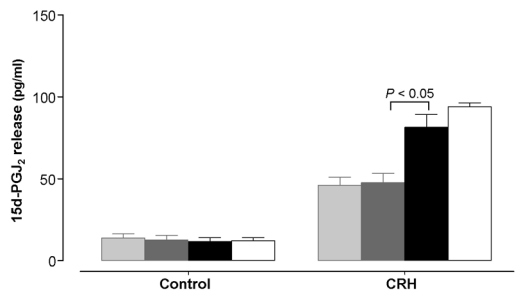


FIGURE 6

Probiotic effect on CRH-induced mast cell degranulation is strain specific

RBL mast cells were pretreated for 1 hr with the individual probiotic strains of which the probiotic mixture is composed ($25x10^5$ CFU/ml). Mast cells were subsequently stimulated with CRH (100nM) and beta hexosaminidase release was measured. Data are mean ± SEM; n=4 preparations from 2 experiments. Comparisons were preformed using ANOVA followed by Tukey's HSD

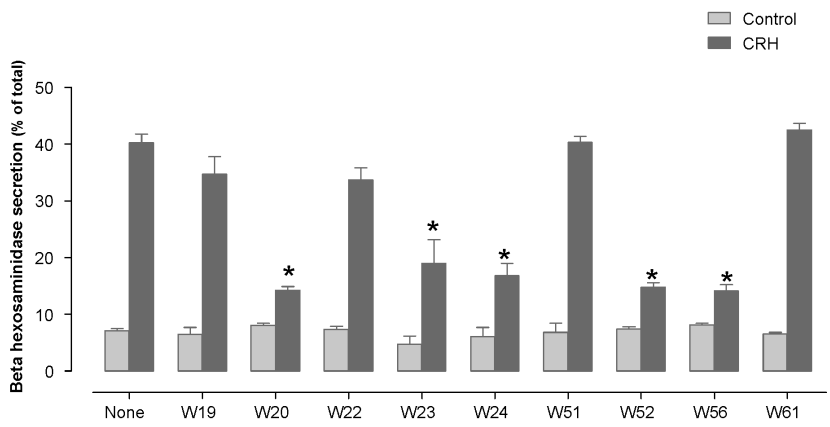


FIGURE 7

CRH-induced mast cell degranulation causes epithelial barrier dysfunction

Bar graph showing that 24 hr exposure of T84 monolayers to CRH-activated mast cells has deleterious effects on (A) transepithelial resistance (TER) and (B) permeability to fluorescent microspheres (0.2 μm). Exposure of T84 monolayers to CRH (100nM) alone did not seem to affect barrier function. Data are mean ± SEM; n=4 preparations from 2 experiments. *, p<0.05 compared to activated, non-treated mast cells. Comparisons were preformed using ANOVA followed by Tukey's HSD.

A / B

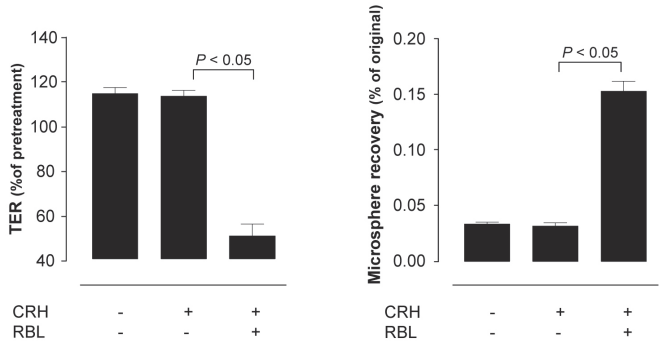
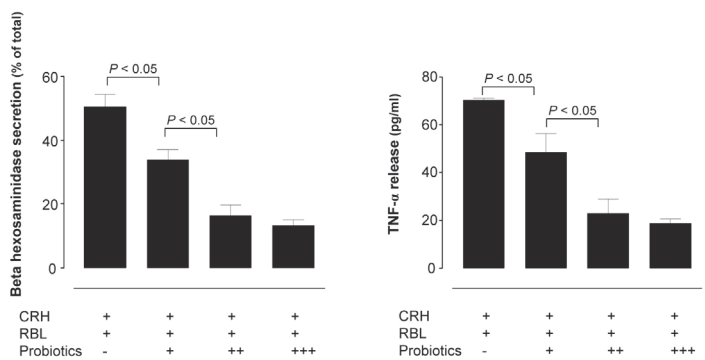


FIGURE 8

Stress-induced increased epithelial permeability is decreased by probiotic pretreatment

Filter-grown T84 monolayers were co-cultured with RBL cells and probiotics were added to the apical side. After 1hr of pretreatment with probiotics, mast cells were activated by addition of 100nM CRH to the basolateral side. Bar graphs showing that pretreatment with probiotics decreases (A) beta hexosaminidase and (B) TNF-α release from mast cells, 30 min after addition of 100nM CRH. (C) Conversely, 15d-PGJ_2 release from CRH-activated mast cells was increased after pretreatment with probiotics. The deleterious effects of CRH- activated mast cells on (D) transepithelial resistance (TER) and (E) permeability to fluorescent microspheres (0.2 μm) were ameliorated after pretreatment with probiotics. Exposure of T84 monolayers to probiotics without addition of activated mast cells did not increase transepithelial resistance (TER) (Panel D) or decreased microphere flux (Panel E). Data are mean ± SEM; n=6 preparations from 2 experiments. Comparisons were preformed using ANOVA followed by Tukey's HSD. Probiotic dosage: + = $25x10^4$ CFU/ml, ++ = $125x10^4$ CFU/ml, +++ = $25x10^5$ CFU/ml

A / B



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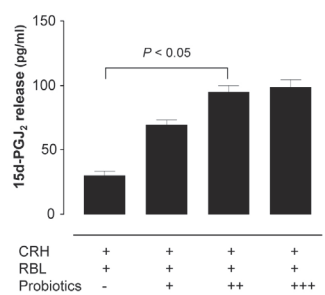


D / E

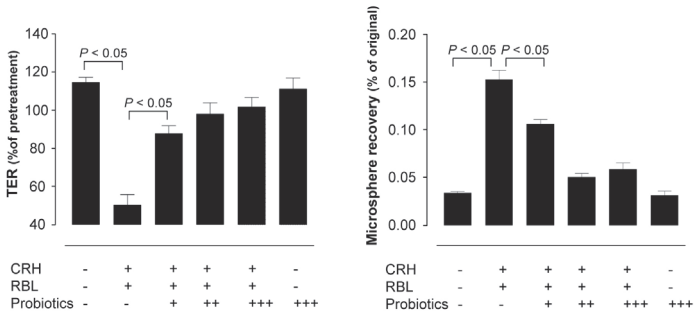

TER (%of pretreatment)
140
120
100
80
60
40
P < 0.05
P < 0.05
CRH - + + + + -
RBL - + + + + -
Probiotics - - + ++ +++ +++
Microsphere recovery (% of original)
0.20
0.15
0.10
0.05
0.00
P < 0.05
P < 0.05
CRH - + + + + -
RBL - + + + + -
Probiotics - - + ++ +++ +++

FIGURE 9

Mast cells contribute to the barrier protective effects of probiotics

Filter-grown T84 monolayers were pretreated with probiotics ($125x10^4$ CFU/ml, 1hr) and either exposed to conditioned medium of CRH activated RBL cells or co-cultured with RBL cells, which were subsequently activated with 100nM CRH. Bar graphs showing that pretreatment with probiotics attenuates the deleterious effects is stress-induced epithelial barrier dysfunction as gauged by (A) an amelioration of the mast cell-induced drop in transepithelial resistance (TER) and (B) increase in microsphere flux after activation of mast cells. In addition, probiotics ameliorated epithelial barrier dysfunction induced by conditioned medium of mast cells, however not to the extent of their effects in the presence of mast cells. Data are mean ± SEM; n=6 preparations from 2 experiments. Comparisons were preformed using ANOVA followed by Tukey's HSD.

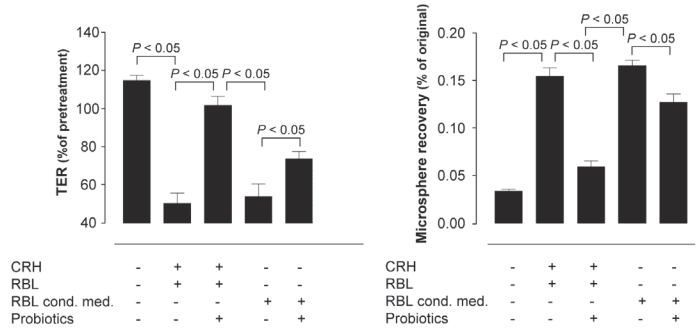


FIGURE 10 A B

Stress-induced increased epithelial permeability is decreased by probiotic soluble factor

Filter-grown T84 monolayers were co-cultured with RBL cells and probiotics ($125x10^4$ CFU/ml, 1hr) or their filter-sterilized supernatant was added to the apical side. After 1hr of pretreatment with probiotics or probiotic supernatant, mast cells were activated by addition of 100nM CRH to the basolateral side. The deleterious effects of CRH-activated mast cells on (A) transepithelial resistance (TER) and (B) permeability to fluorescent microspheres (0.2 μm) were ameliorated after pretreatment with probiotics or their supernatant.

A / B

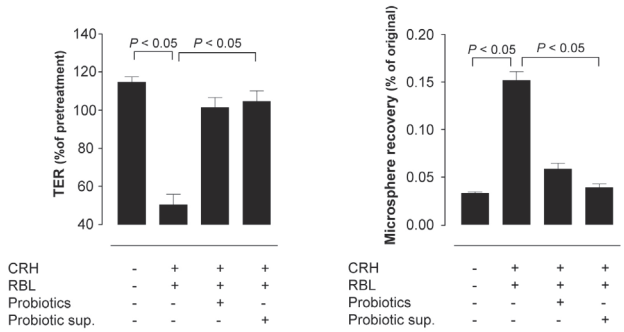
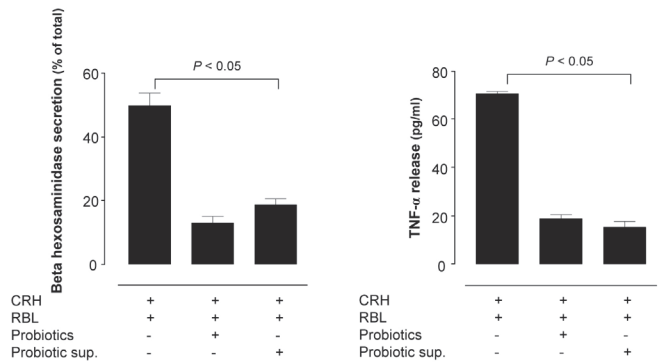


FIGURE 10 C D E

Probiotics and their soluble factors affect mast cells also in co-culture with epithelial cells

Filter-grown T84 monolayers were co-cultured with RBL cells and probiotics ($125x10^4$ CFU/ml, 1hr) or their filter-sterilized supernatant was added to the apical side. After 1hr of pretreatment with probiotics or probiotic supernatant, mast cells were activated by addition of 100nM CRH to the basolateral side. Bar graphs showing that pretreatment with probiotics or their supernatant decreases (C) beta hexosaminidase and (D) TNF-α release from mast cells, 30 min after addition of 100nM CRH. (E) Conversely, 15d-PGJ_2 release from CRH-activated mast cells was increased after pretreatment with probiotics. Data are mean ± SEM; n=6 preparations from 2 experiments. Comparisons were preformed using ANOVA followed by Tukey's HSD.

C / D



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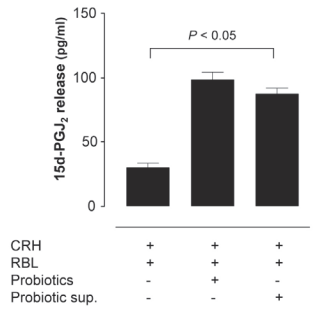


FIGURE 11

Effects of probiotic soluble factors on stress-induced increased epithelial permeability are mast cell-dependent

Filter-grown T84 monolayers were pretreated with probiotics ($125x10^4$ CFU/ml, 1hr) or their filter-sterilized supernatant. After 1hr of pretreatment with probiotics or their supernatant, T84 monolayers were exposed to conditioned medium of CRH-activated mast cells. The deleterious effects of CRH-activated mast cells on (A) transepithelial resistance (TER) and (B) permeability to fluorescent microspheres (0.2 μm) of T84 monolayers were ameliorated after pretreatment with probiotics. However probiotic supernatant had no effect on epithelial barrier dysfunction induced by exposure to conditioned media from CRH-activated mast cells. Data are mean ± SEM; n=6 preparations from 2 experiments. Comparisons were preformed using ANOVA followed by Tukey's HSD.

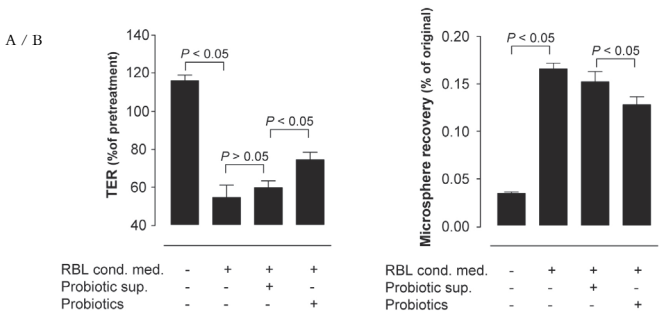
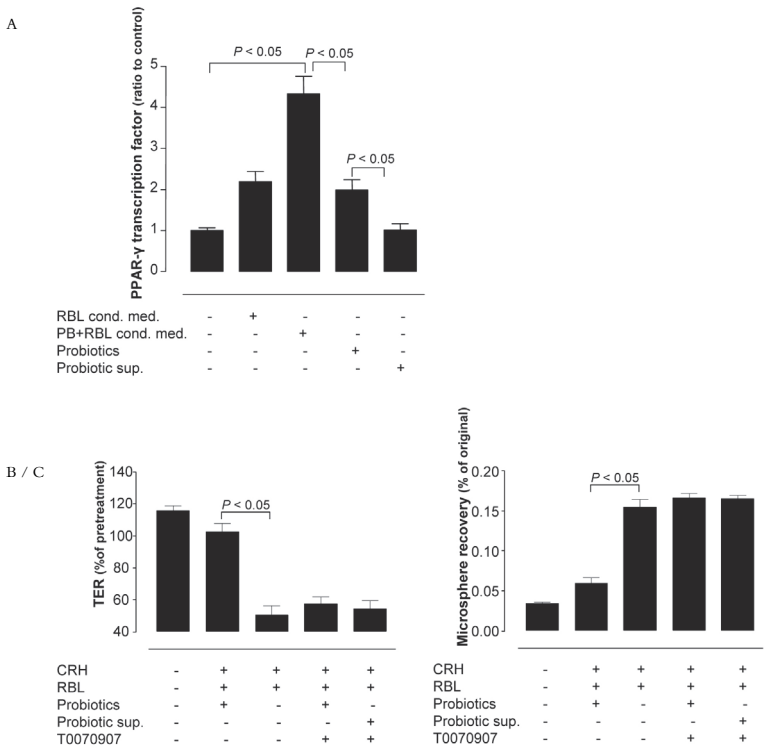


FIGURE 12

Barrier protective effects of probiotics are dependent on PPAR-γ activation

(A) Plastic-grown T84 cells were exposed for 1 hr to 1) conditioned medium of CRH-activated mast cells, 2) conditioned medium of probiotic pretreated ($125x10^4$ CFU/ml, 1hr), CRH-activated mast cells, 3) probiotics ($125x10^4$ CFU/ml, 1hr) or 4) their filter-sterilized supernatant. Bar graph showing PPAR-γ transcription factor levels in nuclear extracts. Data are mean ± SEM; n=4 preparations from 2 experiments. Comparisons were preformed using ANOVA followed by Tukey's HSD. To test whether the barrier protective effects of probiotics were PPAR-γ dependent, filter-grown T84 monolayers were co-cultured with RBL mast cells, incubated with the specific PPAR-γ antagonist T0070907 (5 μM, 30 min) and subsequent pretreated with probiotics ($125x10^4$ CFU/ml, 1hr) or their filter-sterilized supernatant. After 1hr of pretreatment with probiotics or their supernatant, mast cells were activated by addition of 100nM CRH to the basolateral side. Addition of T0070907 abolished the barrier protective effects of probiotics and their supernatant on (B) transepithelial resistance (TER) and (C) permeability to fluorescent microspheres (0.2 μm) of T84 monolayers. Data are mean ± SEM; n=6 preparations from 2 experiments. Comparisons were preformed using ANOVA followed by Tukey's HSD.



DISCUSSION

Mast cells are known to play a pivotal role in stress-induced mucosal barrier dysfunction and release their barrier disruptive content under conditions of stress[23]. The current study expands on previous experiments in a rat model of chronic stress in which our group showed that both in mast cells deficient animals and after inhibition of PPAR-γ, the ability of probiotic bacteria to maintain intestinal barrier function was abrogated[24]. In the current study, we demonstrate that pretreatment with probiotics modulates the mediator profile of mast cells to release significant amounts of 15d-PGJ_2, an endogenous activator of PPAR-γ, resulting in amelioration of barrier dysfunction induced by mast cell activation. Furthermore, the finding that soluble factors released by probiotics exerted similar effects, and sheds more light on the mechanism of action of probiotics.

A number of studies in humans and experimental animals now indicate that stress is associated with abnormalities in intestinal barrier function[25]. Deleterious effects of stress on epithelial function have become more and more established and may explain the proposed role of stress in the pathophysiology of gastrointestinal disorders such as inflammatory bowel disease and irritable bowel syndrome[26]. There is compelling evidence that mast cells regulate barrier physiology and play a key role in stress-induced epithelial responses[27]. Even though, elevated CRH levels (as a natural consequence of stress) have long been associated with increased colonic permeability[28], it was only recently that Wallon *et al.* (2007) showed that human mucosal mast cells indeed express receptors for CRH. Moreover, mast cell stabilizers abolished the effects of exogenous CRH on colonic epithelium[29], strongly suggesting a close link between peripheral effects of CRH, mast cell activation and increased epithelial permeability. In line with these previous findings our results show that rat mast cells (RBLs) indeed can be activated by addition of 100nM CRH, which is a similar concentration of CRH as used to activate the human mast cell line, HMC-1[30]. Furthermore, CRH-induced activation of mast cells was followed by a reduction in TER and increased permeability to microspheres in T84 monolayers. This phenomenon has previously been shown for bacterial peptidoglycan-activated mast cells[31]. However this is the first *in vitro* model using CRH-activated mast cells to inflict epithelial barrier failure, similar to stress-induced mucosal barrier dysfunction.

The ability of the gut to maintain homeostasis requires more than an intact single cell layer of epithelial cells. A functional mucosal barrier should not be considered as a steady state but rather a continuous process of "fine tuning" and the continuous crossing of the epithelial layer of both luminal and endogenous elements illustrates this dynamism. Mast cells are well positioned to participate in the front line immune response to invading pathogens, because of their location

23 Soderholm *et al.*, 2002; Santos *et al.*, 2008; Wallon *et al.*, 2007
24 Lutgendorff *et al.,* Chapter 9
25 Santos *et al.*, 2001; Soderholm *et al.*, 2002; Lutgendorff *et al.*, 2008
26 Bennett *et al.*, 1998
27 Soderholm *et al.*, 2002
28 Santos *et al.*, 1999
29 Santos *et al.*, 1999
30 Cao *et al.*, 2006
31 Wu *et al.*, 2007

and their ability to secrete a large variety of mediators (prostaglandins, cytokines)[32], and to fight of invading bacteria by secreting antimicrobial factors[33]. To fulfill this role though, mast cells must become attuned to differentiating correctly between pathogens versus commensals and respond concordantly. It has been shown that live non-pathogenic *Escherichia coli* inhibit mast cell degranulation[34] and that exposure of mast cells to bacterial products such as lipopolysaccharide or peptidoglycan results in altered mediator production and granular content[35]. Our data show that exposure of mast cells to probiotic bacteria did not lead to degranulation, which is consistent with previous findings[36]. Furthermore, Magerl *et al.* 2008 demonstrated that exposure to commensal bacteria results in inhibition of A23187- and IgE-induced degranulation. Our experiments confirm these findings and show that these results are not restricted to allergy associated IgE-mediated degranulation, but also expand to stress-induced CRH-mediated degranulation. It should be noted that in our experiment incubation with the laboratory strain *E. coli* HB101 did not inhibit mast cell degranulation which is contrary to the results of Magerl *et al.*(2008), suggesting that inhibitory effects of commensals on mast cells are strain specific. This hypothesis is supported by the finding in the current experiments that the individual probiotic strains, also showed strong strain specificity in their effects on mast cell degranulation.

In the present study, pretreatment with probiotics did not only reduce degranulation of mast cells, but also changed the released mediator profile. Mast cell TNF-α release, which has been shown to play a key role in stress-induced alterations of tight junctions and epithelial apoptosis[37], was reduced after pretreatment with probiotics. In contrast, release of 15d-PGJ_2, which has shown to exert barrier protective effects on stress-induced barrier dysfunction[38], was enhanced after exposure to probiotics.

It might be suspected that incubation with lactic acid bacteria may be causative of a reduction in mast cell degranulation due to cell death. The MTT-assay showed that that incubation at all dosage levels, did not affect mitochondrial activity of mast cells and therefore viability can be assumed. It may likewise be thought that reduction in beta hexosaminidase release is an experimental artifact due to degradation of mast cell activators by the probiotic strains. Based on the current results this possibility cannot be ruled out, but is however unlikely since exposure to probiotics increased the release of 15d-PGJ_2.

Previous studies have shown that cell to cell contact with bacteria is necessary to alter degranulation of mast cells in response to activation[39]. Chi *et al.* (2006) found that incubating leukemic human mast cells with heat killed bacteria resulted in secretion of the cytokine MCP-1, but neither cell-free bacterial supernatant nor bacterial lipopolysaccharide had any effect. In our experiments, probiotic supernatant but not dead probiotics affected degranulation and mediator release of CRH-activated mast cells. However in contrast with the previous mentioned study, these

32 Metcalfe *et al.*, 1997
33 Dawicki & Marshall, 2007
34 Magerl *et al.*, 2008
35 Kirshenbaum *et al.*, 2008
36 Magerl *et al.*, 2008
37 Mazzon & Cuzzocrea, 2008
38 Ponferrada *et al.*, 2007
39 Magerl *et al.*, 2008

findings are consistent with other studies showing that bacterial products can modulate and activate mast cells. Leal-Berumen *et al.* (1995) have reported that lipopolysaccharide can induce IL-6 release in rat peritoneal mast cells. Similarly, Marshall *et al.* (1996) have shown that exposure of mast cells to *E. coli* DNA results in secretion of IL-6. Thus cell-to-cell contact with bacteria may or may not be necessary to modulate mast cells depending on the specifics, but our experiments indicate that probiotic derived soluble factors may be responsible for the shown effects. The exact nature of candidate soluble factors however warrants further investigation.

One consequence of the "leaky gut", induced by stress[40] is an enhanced passage of luminal elements such as bacteria or toxins into the internal milieu, either through trans- or paracellular pathways[41]. This may trigger mucosal inflammatory reaction and stimulate the release of pro-inflammatory mediators, further compromising barrier function and initiating a vicious circle. As stress may initiate an early impairment of the barrier function through CRH and mast cell activation, breaking this vicious cycle would be of utmost importance in stress-related gastrointestinal disorders. By 'shifting' the balance between barrier disruptive and barrier protective mast cell mediators, probiotics may contribute to ameliorate stress-induced barrier dysfunction. In the present study, pretreatment with probiotics ameliorated the deleterious effects of CRH-induced mast cell activation on T84 monolayers, in terms of paracellular permeability, as demonstrated by the reduced drop in TER[42], and macromolecular passage, as assessed by the transepithelial flux of microspheres.

These protective effects of probiotics 1) may have been facilitated by modulation of mast cell function 2) are mediated through direct contact with the epithelial cells or 3) are a result of both pathways. Firstly, despite the physical barrier provided by the T84 monolayers between the probiotics on the apical side and the mast cells on the basolateral side, pretreatment with probiotics resulted in alteration of the released mast cell mediator profile after CRH activation as demonstrated by a decreased release of beta hexosaminidase and TNF-α and increased levels of 15d-PGJ_2. These results do not rule out the effects of possible probiotic passage and subsequent direct cell to cell contact. However, as addition of filter-sterilized probiotic supernatant yielded similar effects on mast cell modulation and protection of barrier function, the protective probiotic effects are unlikely to be dependent on bacterial passage through the epithelial layer of probiotics. Furthermore, pretreatment of T84 monolayers with probiotics attenuated barrier disruptive effects of conditioned medium from CRH-activated mast cells, despite the absence of mast cells, although not nearly to the extent of the protective effects exerted by probiotics in the presence of mast cells. This underlines the contribution of mast cells to the barrier protective effects of probiotics.

Secondly, although addition of probiotics to non-stimulated T84 monolayers did not have any effect on TER or permeability to microspheres, the fact that in absence of mast cells probiotics still exerted some protective effects (as mentioned above) suggests that there may also be a direct effect of probiotics on epithelial cells. This is in line with recent studied conducted by Resta-Lenert *et al.* (2009), showing that probiotics exert protective effects on epithelial barrier properties when deranged by

40 Soderholm *et al.*, 2002
41 Nazli *et al.*, 2006
42 McKay *et al.*, 2007

inflammatory cytokines. Interestingly pretreatment with probiotic supernatant could not reproduce these barrier protective effects in the absence of mast cells, suggesting that in direct effects of probiotics on epithelial cells (not involving mast cells), cell-to-cell contact is involved. This is in contrast with the findings of Yan *et al.* (2007), demonstrating that proteins derived from *Lactobacillus rhamnosus* GG prevented TNF-α induced epithelial cell apoptosis and promoted cell growth, which are two key factors in maintenance of epithelial barrier functions. Differences however can be explained by the use of a different probiotic strain as the current study also shows that probiotic effects are highly strain-dependent and can not be extrapolated to other strain or formulations.

Taken together the above, in our experiment protective effects of probiotics on stress-induced epithelial barrier dysfunction are the result of both modulation of mast cell function by probiotics (or rather their soluble factors) and direct effects of probiotics on epithelial cells.

15d-PGJ_2 is a potent PPAR-γ ligand[43] and barrier protective effects of probiotics may be mediated effects on PPAR-γ. PPAR-γ is a major player in maintaining intestinal mucosa homeostasis[44]. Regulating PPAR-γ activity proved effective in treating inflammatory disorders because PPAR-γ interferes with the activity of pro-inflammatory transcription factors and kinases to inhibit cytokine and chemokine production. Previous data indicate that commensal gut bacteria can enhance PPAR-γ activity in intestinal epithelial cells. Microbes are known to influence PPAR-γ activity through pathways involving several factors including, conjugated linoleic acid and H_2O_2, which are both products of probiotic bacteria. In line with these previous findings, our results show that exposure of epithelial cells to probiotics enhances PPAR-γ activity. The finding that incubation with probiotic supernatant did not affect PPAR-γ activity may suggest that close contact between probiotic bacteria and epithelial cells is necessary for this effect on PPAR-γ. However the most dramatic increase in PPAR-γ activity was measured after exposure of T84 cells to conditioned medium of probiotic-treated, CRH-activated mast cells. This supports the hypothesis that probiotics modulate mast cell modulator release resulting in increased levels of 15d-PGJ_2 and enhanced epithelial PPAR-γ activity, ultimately resulting in maintenance of the mucosal barrier function. Finally the finding that addition of the PPAR-γ antagonist T0070907 abolished the protective effects of probiotics suggests PPAR-γ plays a pivotal role in the barrier protective effects of probiotics.

It should be noted that the used *in vitro* model for stress-induced barrier dysfunction consisted of mast cells that originated from rats and a human colonic epithelial cell line. Therefore the data as demonstrated in the current study are the result of factors that are species-unspecific. Furthermore, the beneficial effects of 15d-PGJ_2 may have been overestimated as this is a species-unspecific factor, whereas the deleterious effects of TNF-α may have been suppressed because of its species specificity. However, human and mouse TNF-α has only been shown to behave remarkably similarly and only a small degree of species-specific preference was revealed[45].

43 Forman *et al.*, 1995
44 Ponferrada *et al.*, 2007
45 Fransen *et al.*, 1986

MAST CELLS RELEASE LESS TNF-α AND MORE 15d-PGJ_2 RESULTING IN MAINTENANCE OF EPITHELIAL BARRIER FUNCTION

OUR DATA SHED MORE LIGHT ON A YET UNEXPLORED MECHANISM BY WHICH PROBIOTICS INFLUENCE IMMUNE RESPONSES

In conclusion, this *in vitro* study confirms our *in vivo* data[46] and sheds more light on mechanisms by which mast cells are involved in protective effects of probiotics. Probiotics and their soluble factors were able to modulate mast cells to release less TNF-α and more 15d-PGJ_2, resulting in activation of PPAR-γ in epithelial cells and maintenance of epithelial barrier function despite stress induced mast cell activation. An immunomodulatory role of probiotics has been suggested in numerous studies. However our data shed more light on a yet unexplored mechanism by which probiotics influence immune responses. The ability of probiotics to modulate mast cells has some important implications. In stress-induced gastrointestinal disorders treatment with probiotics could provide a mechanism by which the pro-inflammatory response and consequent barrier dysfunction may be limited.

46 Lutgendorff *et al.*, Chapter 9

11

General discussion

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GENERAL DISCUSSION

"WHO ARE WE?"

"Who are we?" is a question that has boggled the minds of many great philosophers and scientists. In his quest to trace back the origin of our species, Darwin stated that: *"It is not the strongest of the species that survives, nor the most intelligent, but the one most adaptable to change"*. As mammals have now officially been announced to be "the winners" of the evolutionary race[1], one may assume that "we" may be defined as: most adaptable to change. It has been the constant evolutionary pressure from various threats throughout history that resulted in our sophisticated defense mechanisms, adaptable to combat a wide range of stressors in a number of different environments.

The physiologist Hans Selye (1907-1982) defined the physiological responses to stressors and adapted the term stress from physics and engineering to its current use in medical vocabulary and was the first to use the terms stress and "stress response"[2]. The stress response, consisting of a three-phase process and defined by Selye as "General Adaptation Syndrome", results from the adaptation necessary to overcome situations in which an organism has to choose *fight or flight* to survive (e.g. higher blood pressure, a faster cardiac rhythm, suppression of the digestive processes, re-direction of blood to muscles). Thus, all these changes work in concert to allow the organism to detect the danger as well as to provide the insane energy required to survive, while depriving the organism of energy necessary for other processes[3]. This partially self-destructive stress-response is, from an evolutionary perspective, a small price to pay, as threats to homeostasis used to be hungry predators ready for their meal or other life threatening situations. At the present time, however, we do not lie awake, tossing and turning about predators in dark corners. It is daily worries about work and relations that plague our minds. Regardless of the nature of stress (i.e. physiological or psychological) the response to it is rather similar despite the fact that today's hassles are hardly ever solved by fight or flight.

Any threat to homeostasis, whether physical or psychological results in activation of the hypothalamic–pituitary–adrenal axis (HPA). This activation is followed by a compensatory physiological change or adaptation so that the organism can deal with the threat[4]. The main coordinator in the HPA stress response is the corticotrophin release hormone (CRH), which is released from the hypothalamus into the systemic circulation[5]. This, in turn, causes a series of reactions that result in an accelerated heart rate, dilation of blood vessels for muscles, liberation of nutrients for muscular action and increased blood pressure, all to prepare for violent muscular action[6].

In consequence, stress is a dual phenomenon: a fast and intense response which is essential for survival, but the other side of the coin is that it has deleterious effects on the gastrointestinal tract (as reviewed in chapter 2). As the adverse effects of the stress response can be quite extensive, vertebrates have developed sophisticated

1 Alfaro *et al.*, 2009
2 Selye, 1998
3 Sapolsky, 1996
4 Maier & Watkins, 1998
5 Rock *et al.*, 1984; Dunn & Berridge, 1990
6 Maier & Watkins, 1998

WHO ARE WE?

WE DO NOT
LIE AWAKE
TOSSING AND
TURNING ABOUT
PREDATORS IN
DARK CORNERS

counter mechanisms to defend themselves against the deleterious effects of the stress and to "fine-tune" the stress response. For instance, both anti-inflammatory (chapter 9) and anti-oxidative (chapter 5) defense mechanisms were found to be up-regulated in response to stress or critical illness.

Human eukaryotic cells are vastly outnumbered by the number of bacteria that reside in our gut[7]. Newly developed high-throughput techniques are revealing more and more about their role in the maintenance of homeostasis. Therefore this extraordinary capability to battle various threats is not only chiseled into our genetic makeup, but also very much determined by the interplay between our microbiota and mammalian cells. For this reason we hypothesized that pretreatment with probiotics exerts its beneficial effects in part through enhancement of endogenous defense systems.

"LET THY FOOD BE THY MEDICINE"

The concept of probiotics, a term that literally means "for life", was coined at the turn of the 20th century. Russian Nobel laureate Elie Metchnikoff, in his 7 opus *The prolongation of life*: *Optimistic studies*, proposed the unorthodox hypothesis that oral administration of microbes could have beneficial effects for human beings, especially to treat digestive diseases[8]. Nearly a century later, British microbiologist Roy Fuller suggested that beneficial effects are mediated by an improvement in intestinal microbial balance[9]. The modern definition drafted by joint expert consultation of the Food and Agricultural Organization of the United Nations and the World Health Organization in 2001 defines probiotics as "Live organisms which when consumed in adequate amounts confer a health benefit to the host". As reviewed in chapter 2 a wealth of new studies in both the clinical and basic sciences has sought to determine the safety and efficacy of probiotic therapy in the treatment of gastrointestinal disease. The most commonly studied organisms fall within the genera *Bifidobacterium* or *Lactobacillus*, but also include lactic acid bacteria such as *Lactococcus* and *Streptococcus.* Probiotics can be administered as single strain formulas or multistrain combinations. As each strain has its own array of mechanisms of action, it seems intuitive that probiotic strategies may benefit from microbial diversity. In our studies we used disease specific mixtures of probiotic strains designed and selected, based on their antimicrobial, barrier protective and anti-inflammatory properties *in vitro*[10], or their products such as butyrate (chapter 3) or their soluble factors (chapter 10).

There is a vast growing body of literature on the effects of probiotics, and the bewildering complexity of proposed mechanisms of action have raised a certain level of skepticism in the biomedical research community as a whole. Nevertheless recent advances in the field, including the results within the current thesis, are helping to sort out facts from fiction.

7 Shanahan, 2002
8 Metchnikoff, 1907
9 Fuller, 1989
10 Timmerman *et al.*, 2007

EFFECTS OF PROBIOTICS

Due to the enormous complexity of the intestinal mucosal barrier and the diversity in probiotic strains and their properties, discussing mechanisms of action of probiotics has always been a challenge. The distinctions considered below are somewhat artificial in nature, but are simply presented as a conceptual oversimplified framework by which to consider mechanisms of action of probiotics. The depicted figures summarize the probiotic effects within the lumen (fig 1), on epithelial barrier function (fig 2) and on innate and adaptive immunity (fig 3), studied in this thesis.

EFFECTS OF PROBIOTICS ON DIFFERENT LEVELS OF BARRIER DYSFUNCTION

Effects within the lumen

Probiotics are able to transiently colonize the gastrointestinal tract, increasing the concentration of beneficial bacteria, and thereby creating a balance in the intestinal microbiota to the ultimate benefit of the host. A front line of defense against the deleterious effects of potential pathogens is provided by probiotics that exert a direct antimicrobial effect. For example, certain probiotic strains produce antibacterial products referred to as bacteriocins[11]. These antimicrobial factors inhibit the growth and virulence of enteric bacterial pathogens. Lactic acid–producing probiotics may also exert antimicrobial effects on pathogenic organisms by reducing the local pH of the microenvironment in the intestinal lumen[12]. Such speculation is supported by in vitro studies demonstrating that co-culture of *Lactobacillus* species with pathogens results in reduced growth of the virulent organism in a pH-dependent manner[13]. In chapter 4 we demonstrate that the increase in potential pathogens after the induction of acute pancreatitis in rat duodenal samples was prevented by probiotic pretreatment. The most predominant effect of probiotics on the intestinal microbiota was a reduced abundance of enterococci and *Escherichia coli*, which are potential pathogens.

Quorum-sensing refers to a sophisticated communication system between bacteria[14]. It has been suggested that some strains of probiotics interfere with these systems which may influence gene expression of microbial pathogens reducing their virulence potential. For instance, secreted factors from *Lactobacillus acidophilus* (strain La-5) affect virulence gene expression of enterohemorrhagic *E. coli* of the strain O157:H7. *L. acidophilus* reduces the secretion of autoinducer-2 molecules by the pathogenic *E. coli*, which results in reduced expression of genes that are critical for mediating intimate bacterial binding to host-cell surfaces[15]. Interfering in this system reduces the pathogenicity of unwanted microbes because bacterial adherence to epithelial cells is the first and most important step for potential pathogens on their mission to invade the mucosal barrier. This is in keeping with the results described in chapter 8 in which laser confocal microscopy of ileal tissue revealed that pretreatment with probiotics reduced the adherence and invasion of *E. coli* K12 in rats subjected to acute pancreatitis.

11 Brook, 1999
12 Topisirovic *et al.*, 2006
13 Fayol-Messaoudi *et al.*, 2005
14 Hughes & Sperandio, 2008
15 Russell *et al.*, 2007

FIGURE 1

Schematic depiction of probiotic effects within the lumen addressed in this thesis

Probiotics reduce bacterial overgrowth, and decrease adherence of potential pathogens, while maintaining to be able to adhere to the mucosal surface themselves.

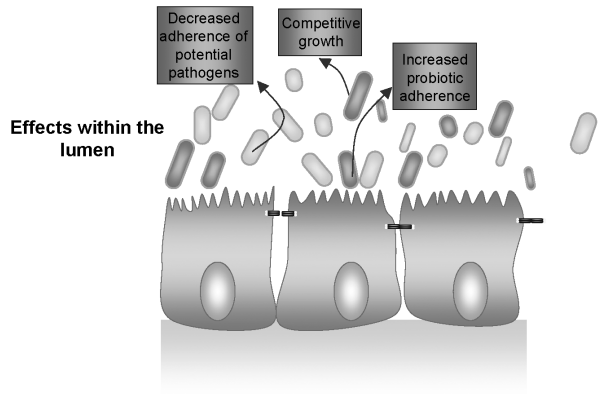


FIGURE 2

Schematic depiction of probiotic effects on the epithelial barrier addressed in this thesis

Tight junctions remain intact and epithelial cell apoptosis is prevented, thereby maintaining barrier function and preventing translocation of macromolecules under conditions of stress and acute pancreatitis.

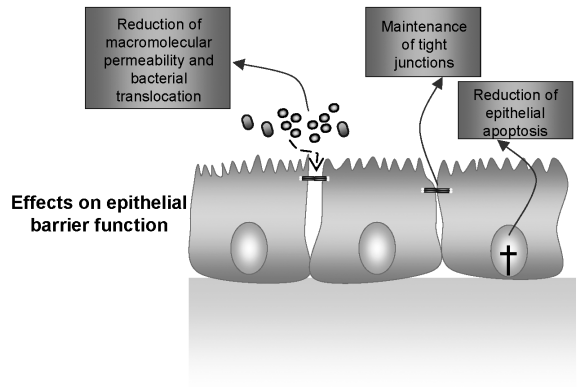
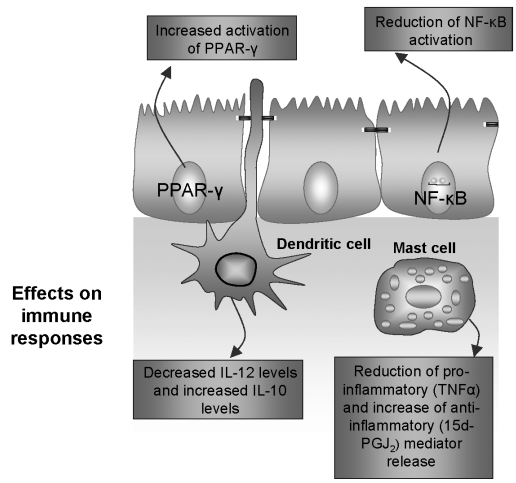


FIGURE 3

Schematic depiction of probiotic effects on the innate and adaptive immune system addressed in this thesis

Butyrate (short chain fatty acid produced by probiotic bacteria) possess the ability to inhibit activation of the pro-inflammatory signal; nuclear factor (NF)-κB. Furthermore probiotics modulate mast cells to enhance secretion of 15-deoxy-Δ 12,14-prostaglandin J_2 (15d-PGJ_2) and reduce secretion of tumor necrosis factor (TNF)-α, modulate dendritic cells to express more interleukin (IL)-10 and less IL-12 and increase peroxisome proliferator-activated receptor (PPAR)-γ activity in epithelial cells.



Probiotics may also compete for a limited niche in the complex microbiota, thereby excluding a site for replication by pathogens[16]. This mechanism of action is often referred to as colonization resistance. In chapter 9 we demonstrate that the increase in adherent *Lactobacillus* species after pretreatment with probiotics coincided with a decrease in bacterial adherence of potential pathogens in rats subjected to chronic stress.

Effects on epithelial barrier function

In addition to promoting the production of antibacterial substances, probiotics also have a direct effect on enhancing the integrity of the epithelial barrier, which serves as the surface lining of the gastrointestinal tract (fig 2). Probiotics may do so by enhancing the electrical resistance of tight junctions contained in the apical junctional complexes between the adjacent polarized epithelia[17]. Indeed, soluble factors secreted by probiotics reduced barrier permeability induced by activation of mast cells in T84 epithelial cell monolayers in an *in vitro* model of stress-induced barrier dysfunction (chapter 10). Of note, exposure of T84 monolayers to probiotics did not further increase transepithelial electrical resistance as compared to naïve monolayers. However, also factors produced by probiotics or commensal bacteria can have protective effects on epithelial barrier properties. In the same experiment, soluble factors derived from the studied probiotics, also showed beneficial effects on mast cell-induced epithelial barrier dysfunction. Furthermore, butyrate, a short-chain fatty acid produced by probiotics and other commensal bacteria[18], was able to reduce bacterial translocation across metabolically stressed epithelial cells, induced by exposure to dinitrophenol (chapter 3).

The integrity of tight junction proteins (such as occludins and claudins) is a key factor in a properly functioning epithelial barrier[19] and prevents invasion of luminal content via paracellular routes. In chapter 5 immunohistochemistry demonstrated that pretreatment with probiotics prevented the devastating effects of a critical illness such as acute pancreatitis on tight junction proteins.

Another determining factor in barrier function is programmed cell death (apoptosis) of the epithelia cells[20]. In spite of apoptosis being a physiological event in epithelial cells, an increased apoptotic rate can cause the barrier to breach[21]. Therefore decreased apoptosis may serve to maintain epithelial barrier integrity and resistance to injurious agents, including enteric pathogens. Chapter 5 did indeed show a positive correlation between mucosal DNA-fragmentation (an accepted index of apoptosis) and barrier dysfunction, supporting this hypothesis.

Finally and most importantly, probiotics showed tangible effects on barrier function *in vivo.* Ussing chamber studies demonstrated that stress both of physical (chapter 5 & 8) and psychological (chapter 9) nature affected mucosal barrier function quite extensively. Pretreatment with probiotics had beneficial effects in both models of barrier dysfunction and on all the tested markers including the passage of *E. coli*. This attenuation of permeability to bacteria was also reflected in reduced

16 Wu *et al.*, 2008
17 Sherman *et al.*, 2005
18 Sakata, 1987
19 Van Itallie & Anderson, 2006
20 Gitter *et al.*, 2000
21 Bojarski *et al.*, 2001

numbers of translocating bacteria from the intestinal lumen to extraintestinal sites. Both in acute pancreatitis-subjected rats (chapter 4) as well as in animals subjected to stress (chapter 9) pretreatment with probiotics reduced bacterial translocation as determined by microbiological cultures of extraintestinal organs.

Effects on innate and adaptive immune responses

Probiotics are able to affect both the innate and adaptive arm of the host immune system[22] (fig 3). These immunomodulatory properties may be one of the most studied features of probiotics and mechanisms include increased phagocytosis capacity, enhanced cell mediated immunity, and stimulation of natural killer cells[23].

By affecting the innate immune system probiotics enhance mucosal barrier properties. For instance, some probiotic strains have the ability to increase the production of secretory immunoglobulin (Ig) A[24]. IgA sticks to the mucus layer overlying the intestinal epithelium and binds to potential pathogens, thereby limiting their ability to gain access and bind to epithelial cells. Probiotics can also prevent activation of the pro-inflammatory nuclear transcription factor, nuclear factor (NF)-κB[25]. This results in decreased secretion of interleukin (IL)-8, which is a potent neutrophil chemoattractant, thereby enhancing inflammation. A major limiting factor in NF-κB activation is the presence of the inhibitor of NF-κB: I- κB. Probiotics have been shown to prevent NF-κB activation by blocking degradation of I-κB[26]. This is in line with our findings in chapter 3 in which treatment with butyrate prevented NF-κB activation in metabolically stressed epithelia through reduction of I-κB degradation. These findings coincided with amelioration of *E. coli* passage through the epithelial layer which emphasizes the importance of "fine-tuning" of the immune response by probiotics and commensals to maintain barrier function.

Another part of the innate immune system is activation of mucosal mast cells. Mast cells play a pivotal role in stress-induced mucosal barrier dysfunction because of their activation via CRH and subsequent release of barrier disruptive content[27]. Previous data had already shown that commensals can influence mast cell degranulation[28]. However, in chapter 10 we demonstrate for the first time that probiotics modulate mast cell mediator release. After pretreatment with probiotics mast cells released lower levels of tumor necrosis factor (TNF)-α and increased levels of 15-deoxy-Δ-12,14-prostaglandin J_2 (15d-PGJ_2) (a prostaglandin derivative with potent anti-inflammatory properties[29]). This changed the released mast cell content from being primarily barrier disruptive to being barrier protective, resulting in amelioration of barrier dysfunction induced by mast cell activation. The differential results of experiments with the individual strains emphasize further the strain-specific effects of probiotics on the host.

Certain adaptive immune responses, which are mainly orchestrated by dendritic cells, are also affected by probiotics. For example, experiments described in chapter 8

22 Walker, 2008
23 Delcenserie *et al.*, 2008
24 Corthesy *et al.*, 2007
25 Fiocchi, 2006
26 Neish *et al.*, 2000
27 Wallon *et al.*, 2007
28 Magerl *et al.*, 2008
29 Tontonoz & Spiegelman, 2008

demonstrated that after pretreatment with probiotics dendritic cells in rats subjected to acute pancreatitis did not express the same high levels of the pro-inflammatory cytokine IL-12 as compared to placebo treated rats. Furthermore, pretreatment with probiotics resulted in increased mucosal levels of the anti-inflammatory cytokine IL-10. The relative levels of IL-10 and IL-12 in particular are of paramount importance, as this determines the nature of T-cell response to be pro-inflammatory or regulatory. Therefore this may be of clinical importance. And indeed, the relative secretion profiles are reported to predict the ability of probiotic strains to have an impact on disease outcome[30]. In humans with irritable bowel syndrome (IBS) for instance, participants randomized to treatment with *Bifodobacterium infantis* (strain 35624) resulted in an improvement in clinical symptoms in association with a normalization of the ratio of IL-10:IL-12 secretion by peripheral blood mononuclear cells[31].

EXTRA-INTESTINAL EFFECTS OF PRETREATMENT WITH PROBIOTICS

As we are only beginning to appreciate the functional significance of the intestinal microbiota, including their effect on human physiology and disease[32], new evidence has emerged that provides a fresh perspective on treatment options for a wide range of acute and chronic disorders. Evidence suggests that perturbations of the gastrointestinal microbiota underlie many diseases and therefore manipulation of the microbial communities has the potential to ameliorate different gastrointestinal diseases[33]. The composition of the microbiota may also affect physiology in extraintestinal compartments (effects studies in this thesis are summarized in fig 4).

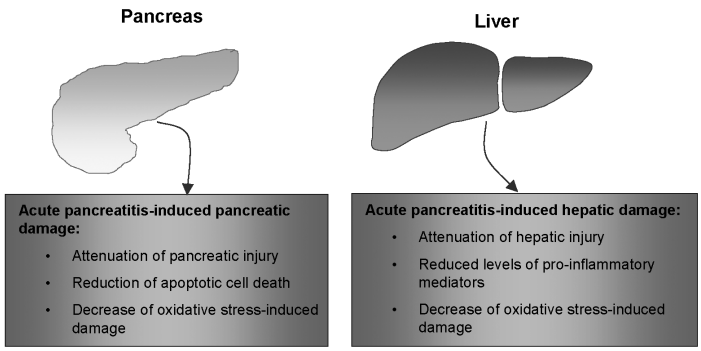
Effects on pancreatic injury

In chapter 6 our group demonstrated that the devastating effects of acute pancreatitis on the pancreas were ameliorated after pretreatment with probiotics. In a rat model of acute pancreatitis administration of probiotics attenuated acinar cell injury and the local inflammatory response in the pancreas, putatively via a reduction in oxidative stress levels. Early in the course of acute pancreatitis locally generated oxygen free radicals cause ATP depletion in acinar cells[34]. As apoptosis is an ATP-dependent process, depletion of ATP leaves a damaged cell with no other choice than to follow the path leading towards necrosis[35]. This results in a pro-inflammatory response which was demonstrated by increased levels of interleukin-1β converting enzyme (ICE) and activated NF-κB (chapter 6), leading towards a vicious cycle as pro-inflammatory signals attract more oxygen free radical generating neutrophils causing more tissue damage[36]. The mechanism of action of this probiotic-induced resilience against oxidative stress, putatively relates to a systemic enhancement of the anti-oxidative capacity, which will be discussed below. Other effects of alteration

30 Foligne *et al.*, 2007
31 O'Mahony *et al.*, 2005
32 Gill *et al.*, 2006
33 Guarner *et al.*, 2006
34 Rau *et al.*, 2000
35 Lemasters, 2005
36 Gukovskaya *et al.*, 1997

FIGURE 4

Schematic depiction of extra-intestinal probiotic effects addressed in this thesis



of the microbiota on the pancreas have been reported in literature. For example, intestinal microbiome composition represented a key epigenetic factor modifying predisposition to type-1 diabetes in the non-obese diabetic mouse model[37].

Effects on liver injury and its inflammatory response

Liver dysfunction and failure contribute to the high mortality rates seen in patients with Gram-negative sepsis, in which mucosal barrier dysfunction is a common problem. The gastrointestinal tract appears to be a key player in the pathogenesis of liver failure in septic models as the high systemic levels of TNF-α induce a breakdown of intestinal barrier function[38]. The liver is the first organ to be subjected to and to suffer the deleterious effects from substances and bacteria that breach the barrier and drain via the portal circulation from the gastrointestinal tract to the liver. The presence of bacteria or bacterial DNA in the liver causes an inflammatory reaction, resulting in further increase of systemic TNF-α levels and causing the system to enter a vicious cycle[39]. As discussed above, probiotic bacteria play a vital role in maintenance of the intestinal barrier function and in chapter 4 a reduction in bacterial translocation was demonstrated after probiotic pretreatment in a rat model of acute pancreatitis. In chapter 7 we hypothesized that pretreatment with probiotics would also be effective in preventing liver injury and attenuating hepatic cytokine secretion in an acute pancreatitis model. In agreement with this hypothesis, our findings demonstrated that administration of probiotics prevented acute pancreatitis–induced liver injury as gauged by ameliorated liver histopathology scores, diminished levels of apoptotic markers (caspase 3 and DNA-fragmentation) and a reduction in systemic levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Supporting the hypothesis that hepatic injury results in an amplification of the pro-inflammatory signal; reduced levels of ICE and activated NF-κB were found concurrent with ameliorated hepatic injury after probiotic pretreatment. Furthermore, both mediators released from the pancreas and from the intestinal tract may damage the liver during the course of acute pancreatitis. Correlation analysis demonstrated that parameters of mucosal barrier dysfunction (as described in chapter 5) correlated better with liver injury scores and hepatic levels of pro-inflammatory mediators than factors of pancreatic damage (chapter 6). This finding suggests that mucosal barrier dysfunction may be a key pathophysiological factor in liver injury, emphasizing the potential role of probiotics in limiting the extent of hepatic injury. This hypothesis is not only appreciated in the context of critical illness but also in the context of chronic liver diseases in which probiotics and improved barrier function have shown promising results[40].

The above findings, taken together, suggest that directed manipulation of the microbiome within the gastrointestinal tract yields benefits at remote sides, which is supported by numerous clinical trials. For instance, pregnant women consuming oral probiotics conferred protection to their infants by reducing the risk of atopic eczema[41] and children treated with *Lactobacillus rhamnosus* GG showed a reduced

37 Wen *et al.*, 2008
38 Akashi-Takamura *et al.*, 2006
39 Hassoun *et al.*, 2001
40 Mencin *et al.*, 2009
41 Kukkonen *et al.*, 2007

TODAY'S HASSLES ARE HARDLY EVER SOLVED BY FIGHT OR FLIGHT

THIS EXTRAORDINARY CAPABILITY TO BATTLE VARIOUS THREATS IS NOT ONLY CHISELED INTO OUR GENETIC MAKEUP, BUT ALSO VERY MUCH DETERMINED BY THE INTERPLAY BETWEEN OUR MICROBIOTA AND MAMMALIAN CELLS

risk of atopic eczema during a 4-year follow up period[42]. Furthermore, probiotics have also been shown to colonize the genitourinary tract after oral administration[43] and demonstrate promising effects in the context of neurological and psychiatric diseases[44].

THE EFFECTS OF PRETREATMENT WITH PROBIOTICS ON ENDOGENOUS DEFENSE SYSTEMS

When timing is the name of the game

Several clinical studies have investigated the potential beneficial effects of probiotics in hospitalized patients. Critically ill hospitalized patients can be divided into two main categories: patients that already are in a critically ill state on admission (e.g. multi trauma patients, severe acute pancreatitis) and patients that will undergo major elective abdominal surgery. In the latter category 10 randomized controlled trials have been conducted so far which showed various results, ranging from no significant effects to up to 93% reduction in post-operative infection rates[45]. However, no adverse effects of probiotics were demonstrated amongst these surgical patients.

Patients undergoing elective surgery with a high risk of bacterial infection, for instance liver transplantation or pancreatic surgery, benefited most from probiotic administration. Even though the precise extent of the critical illness resulting from the operative procedure cannot be predicted, the time of induction of the illness is known precisely, making it possible to start treatment even before the critical illness occurs; this is a treatment strategy that some of the investigators have applied. Up to now, seven studies have been published that enrolled these high risk patients (table 1). Interestingly, two out of the four studies in which patients were only treated postoperatively, failed to yield any significant reduction of bacterial infections after probiotic treatment. On the other hand, all three of the studies that did take advantage of the possibility of starting probiotic treatment pre-operatively, were able to significantly reduce postoperative bacterial infections. Moreover, Sugawara and colleagues (2006) demonstrated that consecutive pre-operative and post-operative probiotic treatment is more effective in reducing post-operative infectious complications as compared to post-operative treatment alone. Additionally, recent experimental work supports this hypothesis. Employing an acute pancreatitis model in mice Rychter *et al.* (2009) were able to show that pretreatment with multi-species probiotics ameliorated AP-induced intestinal barrier dysfunction, while probiotic treatment after induction of AP did not exert any effects, concluding that the efficacy of probiotics depends on timing of administration.

These beneficial effects draw sharp contrast with the results recently published by the Dutch Acute Pancreatitis Study Group[46]. In a nationwide, multi-center trial, administration of probiotics almost doubled the mortality in predicted severe acute pancreatitis patients. In this study, 298 patients were enrolled and treated with probiotics or placebo within 72 hours after onset of symptoms. In addition to an

42 Kalliomaki & Isolauri, 2003
43 Anukam *et al.*, 2006
44 Parr, 2008
45 Rayes *et al.*, 2002
46 Besselink *et al.*, 2008

increase in mortality, probiotic treated patients demonstrated a higher incidence in bowel ischemia (placebo *vs.* probiotics, 0 *vs.* 9 patients). It should be noted that urine analysis demonstrated that probiotic treatment resulted in increased intestinal fatty acid binding protein (IFABP) levels (an accurate marker of intestinal mucosal injury resulting from ischemia[47]) only in patients in whom the course of the disease was complicated by multiorgan failure. Furthermore, treatment with probiotics resulted in an overall reduction in bacterial translocation (as gauged by decreased nitric oxide excretion into the urine) compared to placebo-treated patients[48]. However, subgroup analysis showed that in patients with organ failure bacterial translocation was increased after probiotic treatment, indicating that probiotics had beneficial effects in the moderately ill patients, but by the same token, had deleterious effects in the critically ill.

These unforeseen results might, at first glance, appear to be in contrast with our hypothesis that probiotics enhance endogenous defense systems. However, given that probiotics seemingly have better efficacy as preventive treatment strategy and that the same probiotic combination shows beneficial effects in moderately ill and adverse effects in critically ill patients, it might be envisioned that probiotics themselves may be a minor stressor and thereby may enhance endogenous defense systems (fig 5) under steady state conditions, while causing adverse effects in an already significantly unbalanced system such as, for instance, in critically ill patients.

47 Gollin *et al.*, 1993
48 Besselink *et al.*, 2009

TABLE 1

Effects of probiotic treatment in 7 randomized controlled trials in surgical patients with a high risk of post operative bacterial infections

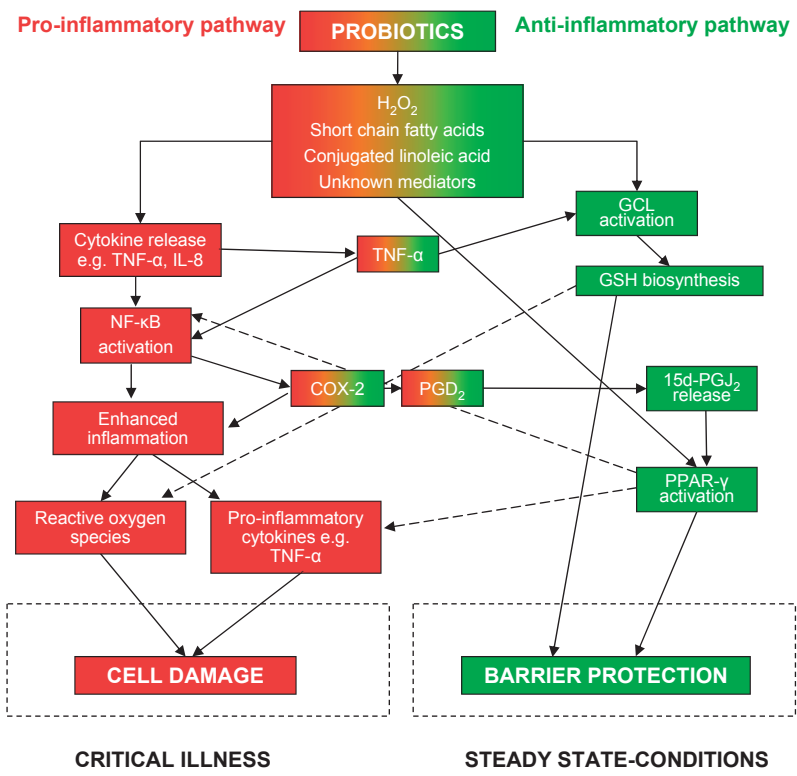
	Category	Length of therapy (days)	Treatment	n=	Control group	n=	Infection rate (Probiotic *vs.* Control)
Post-operative treatment							
Rayes *et al.* 2002a	Major abdominal surgery (liver gastric, pancreas colon)	5 post	10^9 *L. plantarum* 299 + oat fiber	30	10^9 heat killed *L. plantarum* 299 + oat fiber	30	10% *vs.* 10% (n.s.)
Rayes *et al.* 2002b	Liver transplantation	12 post	10^9 *L. plantarum* 299 + oat fiber	31	10^9 heat killed *L. plantarum* 299 + oat fiber	32	13% *vs.* 34% (n.s.)
Rayes *et al.* 2005	Liver transplantation	14 post	Synbiotic 2000 (10^{10} of 4 different LAB and 4 fibers)	33	Fibers only	33	3% *vs.*48% (*P*=<0.0001)
Kanazawa *et al.* 2005	Hepatectomy	14 post	10^8 *B. breve,* 10^8 *L. casei* + enteral feeding	21	Enteral feeding	23	19% *vs.* 52% (*P*=0.03)
Peri-operative treatment							
Rayes *et al.* 2007	PPPD	1 pre 8 post	Synbiotic 2000 (10^{10} of 4 different LAB and 4 fibers)	40	Fibers only	40	12.5% *vs.* 40% (*P*=0.01)
Nomura *et al.* 2007	PPPD	3-15 pre 10 post	*E. faecalis C. butyricum, Bacillus mesentericus*	30	No treatment	34	23% *vs.* 53% (*P*=0.02)
Sugawara *et al.* 2006	Hepatectomy	14 pre 14 post	10^{10} *B. breve,* 10^{10} *L. casei* (perioperatively)	41	10^{10} *B. breve,* 10^{10} *L. casei* (post operatively only)	40	12.1% *vs.* 30% (*P*=0.049)

PPPD, pylorus-preserving pancreaticoduodenectomy; post, post-operatively; pre, pre-operatively; *L., Lactobacillus; B., Bifidobacterium; E., Enterococcus; C., Clostridium*. p-values were calculated using Fisher's exact test.

FIGURE 5

Overview of probiotic interactions with endogenous defense mechanisms

Inside the intestinal lumen, probiotics produce mediators active in immune signaling, including hydrogen peroxide (H_2O_2), short chain fatty acids and conjugated linoleic acid. Hydrogen peroxide can cause a pro-inflammatory reaction in epithelial cells, resulting in the release of cytokines and activation of nuclear factor (NF)-κB. Activation of NF-κB will in turn lead to activation of cyclooxygenase (COX)-2, which will further enhance the inflammatory signal, but also induce anti-inflammatory effects via induction of prostaglandin (PG)D_2 and 15-deoxy-Δ 12,14-prostaglandin $_{J2}$ (15d-PGJ_2). The latter is the endogenous activator of peroxisome proliferator-activated receptor (PPAR)-γ, which has known anti-inflammatory and barrier protective properties. In addition, cytokine release e.g. release of tumor necrosis factor (TNF)-α, results in increase in glutamate cysteine ligase (GCL) activity, resulting in enhanced biosynthesis of glutathione (GSH), which is the major endogenous anti-oxidant and protects against oxidative stress. On the other hand, hydrogen peroxide, produced by probiotics can also directly activate GCL and result in GSH biosynthesis. Furthermore, conjugated linoleic acid is known to directly activate PPAR-γ. This thesis hypothesizes that in steady state conditions probiotics will result in enhanced endogenous defense systems (i.e. enhanced PPAR-γ activation and GSH biosynthesis). In a critically ill state, however the enhancement of these defense systems is too little too late and any extra pro-inflammatory signaling will cause further inflammation and epithelial cell damage, resulting in intestinal barrier dysfunction. Straight lines represent stimulatory signaling and dotted lines represent inhibitory signaling.



Effects on anti-oxidative defenses

In chapter 5 we demonstrated that probiotic preconditioning resulted in enhanced mucosal levels of the major endogenous anti-oxidant, glutathione. Mucosal glutathione content is mainly dependent on dietary intake and *de novo* synthesis. The latter is in turn associated with availability of cysteine (the rate-limiting amino acid in glutathione synthesis) and activity of glutamate-cysteine-ligase (which plays a pivotal role in the formation of glutathione from cysteine and glutamate). Even though all bacteria tested positive for glutathione, their content was so minute that bacterial glutathione could only partially account for the rise in mucosal glutathione. Furthermore, cysteine availability in probiotic treated rats was unchanged as compared to placebo treated animals, and therefore the increased activity of glutamate-cysteine-ligase is putatively the mechanism underlying the increase in mucosal glutathione. This hypothesis was supported by the demonstrated increase in mRNA expression of the two subunits of glutamate-cysteine-ligase. Glutamate-cysteine-ligase gene expression is up-regulated after a low dose of H_2O_2[49] and after administration of weak inducers of oxidative stress[50]. Therefore the increased glutathione biosynthesis could be indicative of a previous cellular stressor provided by the administration of probiotics. As probiotics are producers of short-chain fatty acids which reduce the pH and induce epithelial cell production of stimulatory cytokines, one can imagine them to have a minor cytotoxic effect. In addition, there is evidence showing that certain lactic acid bacteria produce H_2O_2[51].

Not only did probiotic pretreatment enhance glutathione levels locally in the mucosa, but administration of probiotics resulted in both enhanced systemic glutathione biosynthesis (as gauged by increased gluatamate-cysteine-ligase activity in red blood cells, chapter 6) as well as enhanced glutathione levels in liver (chapter 7) and pancreas (chapter 6). However, exact mechanisms on how probiotics enhance the anti-oxidative defense and the mediators involved warrant further investigation before this probiotic mechanism of action can fully be employed in a clinical setting. The finding that glutathione levels correlated well with reduced injury scores in the ileum (chapter 5), pancreas (chapter 6) and liver (chapter 7) as well as with the cytokine response generated by the liver, underlines the importance of a "preconditioning" of this antioxidant defense mechanism.

As oxidative stress is a very early event in acute pancreatitis[52], the therapeutic potential of this action of probiotics is limited in acute pancreatitis patients after the onset of symptoms. However there are numerous other clinical circumstances in which a period of (intestinal) oxidative stress can be predicted and therefore prevented. Examples are elective major abdominal surgery in which probiotics have already shown promising effects with respect to attenuation of post-operative infections[53], patients undergoing endoscopic retrograde cholangiopancreatography, in which acute pancreatitis is one of the major complications or maintenance treatment in inflammatory bowel patients[54], including patients suffering from pouchitis[55]).

49 Ding *et al.*, 2008
50 Solis *et al.*, 2002
51 Voltan *et al.*, 2008
52 Reinheckel *et al.*, 1999
53 Sugawara *et al.*, 2006
54 Kruis *et al.*, 2004
55 Gionchetti *et al.*, 200

Effects on anti-inflammatory defenses

To combat potential threats to homeostasis, the body reacts by activation of the immune system of which the cardinal task is to eradicate and eliminate hostile pathogens. However, by doing so, collateral damage to the body's own tissue is inevitable. The total damage therefore is not only determined by the injury inflicted by the pathogen, but very much so determined by the self-inflicted injury of the immune system. It has only recently been appreciated that resolution of the inflammatory state is an active process, and that inflammation has to be actively put to a stop to limit the self-inflicted collateral damage. One of the mechanisms of interest is the role of certain prostaglandins as these are considered as a possible endogenous regulator of the inflammatory response in neurodegenerative conditions. One of these prostaglandins is the anti-inflammatory prostaglandin 15d-PGJ_2, a structural, non-enzymatic derivative of the prostaglandin D_2 and the endogenous ligand for peroxisome proliferator activated receptor (PPAR)-γ[56]. Activation of this nuclear receptor has been associated with numerous anti-inflammatory effects, including inhibition of NF-κB activation. Furthermore, PPAR-γ has been shown to pay a crucial role in maintenance of barrier function in a chronic stress model[57] and recently the gene encoding for it has been identified as a susceptibility gene in Crohn's disease[58].

Mucosal mast cells are major sources of prostaglandins in the intestines and hypothetically may exert barrier protective properties. However, mast cells are infamous for their role in mucosal barrier dysfunction under stress[59]. Nevertheless, we hypothesized that probiotics may modulate mast cell mediator release and thereby change its role from being primarily deleterious to being protective under chronic stress conditions. In chapter 9 in a rat model of chronic stress, pretreatment with probiotics resulted in enhanced 15d-PGJ_2 levels, increased mucosal PPAR-γ activation and attenuation of the stress-induced mucosal barrier dysfunction and mucosal inflammation. In mast cell deficient animals however, probiotic pretreatment only slightly increased PPAR-γ activation and did not result in enhanced levels of 15d-PGJ_2. Most importantly, in mast cell deficient animals probiotic pretreatment did not prevent mucosal barrier dysfunction, indicating that the protective effects of probiotics are dependent on mast cells. Furthermore, injection of the animals with the specific PPAR-γ antagonist, T0070907, completely abolished the beneficial effects of probiotics on barrier function and mucosal inflammation. Based on those data however it was impossible to distinguish whether the protective effects afforded by probiotics were entirely dependent on PPAR-γ activation through mast cell derived 15d-PGJ_2 or that beneficial effects are also mediated through a direct probiotic induced activation of PPAR-γ in epithelial cells. Therefore an *in vitro* experiment was conducted. Findings in chapter 10 indicate that besides the above mentioned decreased TNF-α release and increased 15d-PGJ_2 release from mast cells, probiotics cause activation of epithelial PPAR-γ mainly through the increased release of 15d-PGJ_2 from mast cells, but also partly through a direct activation of PPAR-γ in epithelial cells. Interestingly, soluble factors of probiotics were indeed able to modulate mast cell mediators to release more 15d-PGJ_2 and to cause a subsequent increase in epithelial PPAR-γ. However, no increase in PPAR-γ

56 Tontonoz & Spiegelman, 2008
57 Ponferrada *et al.*, 2007
58 Sugawara *et al.*, 2005
59 Soderholm *et al.*, 2002

activation could be determined in epithelial cells alone after exposure to probiotic soluble factors. These results may suggest that the mast cell modulating effects are mediated through probiotic soluble factors, although the direct effects of probiotics on PPAR-γ in epithelial cells are mediated through probiotic-cell contact. The precise probiotic mediators involved in PPAR-γ activation are still to be elucidated. Candidate mechanisms of action are short chain fatty acids, e.g. butyrate[60] and conjugated lenoleic acid[61], which are both probiotic metabolites, or through the production of H_2O_2[62], all of which have been shown to up-regulate PPAR-γ. Interestingly, both short chain fatty acids and H_2O_2 are inducers of anti-oxidative defenses as well.

PPAR-γ has also been suggested to exert anti-inflammatory effects through modulation of dendritic cells. Intestinal mucosal dendritic cells are mainly responsible for antigen and bacterial recognition and have the unique capability of stimulating naïve T-cells and shaping their immune responses[63]. Even though this adaptive immune response is a fair bit more sophisticated than other innate immune reactions, 'fine-tuning' of the response it still of utmost importance. PPAR-γ had already been shown to reduce dendritic cell maturation and IL-12 production, which are both signals stimulating a pro-inflammatory Th1 response[64]. These findings are in line with the results described in chapter 8 demonstrating an inverse correlation between mucosal PPAR-γ activation and IL-12 levels in rats subjected to acute pancreatitis. But most importantly, this study showed for the first time that pretreatment with probiotics resulted in enhanced immunohistochemical staining for PPAR-γ in dendritic cells. The hypothesis that probiotics modulate dendritic cell cytokine expression from a pro-inflammatory towards a more regulatory profile, putatively via upregulation of PPAR-γ, was supported by the concomitant finding that IL-12 positive staining in dendritic cells was reduced after probiotic treatment.

CONCLUSION

Ever since the publication of the Dutch Acute Pancreatitis Study Group describing serious adverse events of probiotic treatment[65], probiotics cannot longer be defined as "Live organisms, which when consumed in adequate amounts confer a health benefit to the host". Rather, this definition ought to be rephrased into "microorganisms with health promoting properties, *in the right patient at the right time*". This thesis provides evidence that probiotics, administered before an illness occurs and thus under steady-state conditions, are able to maintain mucosal barrier function and limit disease severity once the illness occurs, putatively via enhancement of endogenous defense systems. This mechanism by which probiotics work is relatively unexplored and underemployed. However, before this mechanism of action can safely be used to prevent disease, the exact nature of the probiotic mediators that exert these effects warrants further investigation. Furthermore, it should be determined if these mediators indeed cause adverse effects in seriously unbalanced systems and consequently need to be avoided in critically ill patients.

60 Wachtershauser *et al.*, 2000
61 Ewaschuk *et al.*, 2006
62 Voltan *et al.*, 2008
63 Kalinski *et al.*, 2000; Pulendran *et al.*, 2001
64 Faveeuw *et al.*, 2000
65 Besselink *et al.*, 2008

Food for thought

There is food for thought in the fact that everyone has inside them microbial communities that are as diverse as any rainforest. As more and more evidence is suggesting that their influence on human physiology stretches beyond our imagination, there is an obvious need to study the interactions between "them" and "us". That is, if one can really distinguish "them" from "us" as they can be considered as our extended self. Therefore the Human Microbiome Project[66] as well as all other research conducted to investigate the wonderful complexity of the intestinal microbiota will lead us into a new era in which the diversity present in the gastrointestinal tract and the functional consequences of that diversity will be more deeply appreciated. Appropriate use of knowledge uncovered by these research efforts will require a thorough understanding of how commensal, probiotic and pathogenic microorganisms interact with one another and their mammalian host. Identification of key factors and signaling mediators will help explain microbe-microbe and microbe-host interactions at a molecular, cellular and systemic level. But most importantly, as evidence shows that the composition of the human microbiota is as individual as a finger print, it requires no stretch of the imagination, to envision that the effects of antibiotics, probiotics, and for that matter all medicine are in great part determined by interactions with intestinal microbes and therefore highly personal. As we progress from the era of relatively simplistic antibiotics-only approaches, understanding of the human microbiome, probiotics and their function will enable researchers to develop personalized approaches in treatment and prevention of disease.

Thus far, all efforts made to unravel the universe of the intestinal microbiota and its effects on human biology, has put humankind's place in the world into a new perspective: a realization that "Who am I?" is impossible to be fully answered, unless we understand who "we" are.

66 Turnbaugh *et al.*, 2007

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12

SUMMARY

Probiotics have long been regarded as "health promoting microorganisms". However, since the publication of serious adverse effects of probiotic treatment in severe acute pancreatitis patients in the beginning of 2008, that definition might have to be rephrased into: "microorganisms with health promoting properties, in the right patient at the right time".

Despite a large body of evidence on mechanisms by which probiotics exert their health promoting properties, the possibility that probiotics are perceived as a minor toxic assault and thereby augment defense systems, protecting the human body against future damage, is relatively new and unexplored. This thesis hypothesizes that pretreatment with probiotics exerts its beneficial effects in part through enhancement of endogenous defense systems.

NO GUTS NO GLORY

Our understanding of the complexity and function of the intestinal microbiota remains in its infancy, although it is clear that an important symbiotic relationship between commensal microbiota and their host has evolved over millions of years. In **chapter 2** the effects of stress on this symbiotic relationship and possible mechanisms of action of probiotics to counteract these deleterious consequences are reviewed. Psychological stress has been recognized by patients as a factor in gastrointestinal diseases, but has long been ridiculed by the scientific community. However, in the last decades compelling evidence has demonstrated tangible, deleterious effects of stress on intestinal microbiota and mucosal barrier function as well as the clinical course of gastrointestinal diseases. Stressful conditions can change the composition of microbiota resulting in reduced abundance of *Lactobacilli* and increased numbers, epithelial adherence and mucosal invasion of potential pathogens. Furthermore, potential pathogens have developed sophisticated systems to 'sense' the condition of their host and their population density. This allows bacteria to make a cost versus benefits assessment of activation of virulence genes and enables bacteria to launch a surprise attack on the host when it is at its weakest and chances of successful opportunism are at its highest. Probiotics are potentially able to counteract the deleterious effects of stress through several mechanisms, including (I) direct antimicrobial activity through production of bacteriocins; (II) competitive exclusion of pathogens by competition for binding sites and nutrition; (III) maintenance of barrier function via protection of tight junction structure and inhibition of epithelial cell apoptosis; and (IV) regulation of pro-inflammatory signals, such as nuclear factor (NF)-κB signaling. These effects are strain specific and can be mediated by direct bacteria-host interactions and/or via soluble factors or probiotic metabolites.

One such probiotic metabolite is the short-chain fatty acid, butyrate, which is a fermentation product of carbohydrates and the primary energy source for colonocytes. In **chapter 3** we hypothesized that butyrate is important in maintenance and regulation of barrier function of colonic epithelium. An *in vitro* model of the colonic epithelial layer was challenged by exposure to dinitrophenol, which uncouples oxidative phosphorylation and induces metabolic stress. Addition of *E. coli* resulted in decreased transepithelial resistance (reflecting paracellular permeability)

and increased permeability to bacteria. Butyrate restored mitochondrial activity of epithelial cells and reduced bacterial translocation across the stressed epithelial layer. However, the reduction in transepithelial resistance was not affected by butyrate, suggesting that paracellular and transcellular permeability are regulated differently, with the latter being the route that bacteria use to breach the barrier, which is putatively regulated by butyrate. Pro-inflammatory signaling, such as the activation of NF-κB, can result in mucosal barrier failure. Indeed, bacterial passage across the epithelial layer was associated with increased NF-κB activation, which was reduced after butyrate treatment. These novel findings underline the importance of the intestinal microbiota and their metabolites in the maintenance of barrier function as a part of the host innate defense strategy. Furthermore, metabolic stress and reduced butyrate metabolism have been postulated as putative mechanisms underlying colitis ulcerosa. Prebiotics (e.g. oligosaccharide substrates, such as oatmeal) could thus be of value to prevent disease relapse in IBD patients, especially so during times of psychological stress, which is known to cause epithelial mitochondrial abnormalities and perturbation of the intestinal microbiota.

In addition, live probiotics can potentially prevent mucosal barrier failure. **Chapter 4** describes the first study to investigate the effects of a multispecies probiotic mixture specifically designed to reduce mucosal barrier dysfunction in critical illness. During acute pancreatitis and other critical illnesses, failure of the mucosal barrier has devastating clinical consequences such as bacterial translocation, sepsis and multi-organ failure. The root of all evil is difficult to determine however; bacterial overgrowth of pathogens, mucosal ischemia due to reduced intestinal blood flow and intestinal pro-inflammatory responses all play a part in the breakdown of the barrier. Therefore six probiotic strains were selected for their antibacterial and immunomodulatory properties. In a rat model of acute pancreatitis, pretreatment with probiotics resulted in reduced bacterial overgrowth of potential pathogens, and attenuated bacterial translocation to extraintestinal sites, as gauged by bacterial cultures (determining live bacteria) and real-time polymerase chain reaction (PCR) of bacterial DNA (determining the total bacterial load of dead and alive bacteria). Interestingly, the average reduction in bacterial translocation was greater when analyzed by culture than by PCR, suggesting that probiotic pretreatment might render the host more capable of killing invaders at distant sites.

WHAT DOES NOT KILL YOU MAKES YOU STRONGER

Owing much to the constant stream of various threats throughout evolution, mammals are equipped with rather sophisticated endogenous defense systems that allow cells to overcome stressful stimuli and adapt to the level of threatening signals from the environment. Here the old adage: 'what does not kill you, makes you stronger' really comes into play, as previous stressful stimuli determine the level of action of the defense systems. This mechanism underlies concepts such as preconditioning and can be exploited to develop preventive treatment strategies.

Anti-oxidative defense

As mentioned above, during a critical illness reduced intestinal blood flow results in mucosal oxidative stress and contributes to intestinal barrier failure. Luckily,

epithelial cells are well equipped with an anti-oxidative defense system in which glutathione is the key player. Glutathione synthesis occurs within epithelial cells and is catalyzed by glutamate-cysteine-ligase. To be able to adapt to environmental threats, glutamate-cysteine-ligase gene expression is under control of exogenous stressful stimuli and has been demonstrated to be enhanced after administration of weak inducers of oxidative stress.

In **chapter 5** rats subjected to acute pancreatitis were pretreated with probiotics, using the same probiotic composition as employed in the previous study. Induction of acute pancreatitis resulted in intestinal barrier dysfunction which was characterized by epithelial cell apoptosis, disruption of tight junctions, and correlated well with levels of mucosal oxidative damage as determined by lipid peroxidation. Pretreatment with probiotics decreased the mucosal permeability to *E. coli,* ameliorated epithelial apoptosis and disruption of tight junctions and attenuated oxidative stress-induced mucosal damage. Furthermore, this study is the first to show that pretreatment with probiotics increases mucosal glutathione levels and stimulates glutathione biosynthesis as gauged by an increase in glutamate-cysteine-ligase gene expression. This increased gene expression could be indicative of cellular stress as a mechanistic factor. Moreover, the importance of this endogenous defense system against oxidative stress was underscored by the positive correlation between mucosal glutathione levels and improved barrier functions. The results of this study are seemingly in contrast with the adverse probiotic effects reported in 2008. In this randomized controlled trial, probiotic treatment resulted in an increased incidence of bowel ischemia in severe acute pancreatitis patients. However, keeping in mind that probiotics may cause low levels of oxidative stress, administration of probiotics after the onset of acute pancreatitis may have been an extra burden in an already critically balanced system, causing oxidative damage and ischemia. Consequently, probiotics cannot be regarded as a defendable treatment option in critically ill patients.

Pretreatment with probiotics did not only increase the mucosal defense against oxidative stress; anti-oxidative defenses were also enhanced at extra-intestinal sites. In the same rat model, pretreatment with probiotics resulted in reduction of pancreatic injury, again stressing the importance of anti-oxidative defense systems. In addition, pretreatment with probiotics ameliorated the local inflammatory response and reduced ATP depletion induced by acute pancreatitis. The latter is of major importance as necrosis is the result of severe cellular damage and depletion of energy. And, indeed, the extent of pancreatic necrosis was also limited after probiotic pretreatment. Furthermore, *de novo* synthesis of glutathione and glutathione levels were increased locally in the pancreas as well as systemically after probiotic pretreatment, which may be a putative mechanism behind the amelioration of pancreatic injury (**chapter 6**).

During critical illness and as a consequence of mucosal barrier failure, luminal toxins and bacteria drain from the intestinal tract via the portal circulation into the liver. As described in **chapter 7,** probiotic pretreatment ameliorated hepatic oxidative induced damage, putatively via augmentation of the anti-oxidative defense system. The liver is not only one of the affected organs in a critical illness, but plays an important role as amplifier of the immune response as it harbors the largest population of fixed macrophages and is seen as a predominant source of pro-inflammatory cytokines. This hypothesis was supported by the positive correlations between hepatic IL-1β-converting enzyme and NF-κB levels and local lipid peroxidation

(indicating oxidative stress-induced damage). Moreover, probiotics reduced hepatic NF-κB activation, which is known to be involved in the genesis of multi organ failure and lung injury in particular. Mediators released from both the pancreas and the intestinal tract may damage the liver in the course of acute pancreatitis. Correlation analysis showed that parameters of intestinal mucosal barrier dysfunction (**chapter 5**) correlated better with liver injury scores and hepatic levels of pro-inflammatory mediators than factors of pancreatic damage (**chapter 6**), suggesting that mucosal barrier dysfunction may be of greater importance for the amplification of inflammatory signals to the liver.

Even though these beneficial effects of probiotics show great promise in the battle against oxidative stress induced injury, it should be noted that excessive oxygen free radical formation is an early event in the course of acute pancreatitis and therefore a difficult therapeutic target. Therefore to fully benefit from the anti-oxidative effects of probiotics, a preventive approach to improve defense against an expected oxidative attack needs to be thought of, such as elective major abdominal surgery, maintenance treatment in inflammatory bowel diseases or preconditioning of patients undergoing liver resection.

Anti-inflammatory defense

Our immune system tends to overreact to any challenge, as too little of a reaction can be fatal. The other side of the coin though is quite extensive collateral damage, which is the price we pay for a vigilant guard. Mammals have evolved sophisticated ways to fine-tune immune responses, limiting collateral damage. An important factor is the activation of peroxisome proliferator-activated receptor (PPAR)-γ, which can limit pro-inflammatory signaling and is involved in modulation of the function of dendritic cells. Mucosal dendritic cells are able to orchestrate intestinal immune responses due to their unique ability to initiate and modulate T-cell responses. This special trait is attributed to their expression of cytokines which is up-regulated upon internalization of bacteria or antigens. Discriminative factors in this respect are IL-12 levels, contributing to a more pro-inflammatory Th1 response, while IL-10 expression favors a regulatory type Th2 response. Although knowledge is accumulating rapidly on the role of dendritic cells in mucosal inflammation, little is known about their role in acute pancreatitis in which intestinal immune responses are determining factors in disease severity. **Chapter 8** describes the first study to provide evidence that upon bacterial adhesion, which is increased after induction of acute pancreatitis, dendritic cells internalize bacteria and express high levels of IL-12, favoring a Th1 type pro-inflammatory response. Interestingly, after pretreatment with probiotics IL-12 expression by dendritic cells was reduced compared to placebo treated animals, while mucosal IL-10 levels were enhanced. Furthermore, probiotic pretreatment resulted in enhanced PPAR-γ levels in mucosal dendritic cells, which has not been previously reported. Therefore, pretreatment with probiotics may create a mucosal environment that is supportive of regulatory type Th2 responses, putatively via upregulation of PPAR-γ levels in dendritic cells.

PPAR-γ activation has also been shown to play a key role in limiting psychological stress-induced intestinal inflammation. During psychological stress, elevated corticotropin-releasing hormone levels cause mast cells to release their barrier disruptive content of pro-inflammatory mediators. By the same token though,

mast cells are also primary sources of the specific endogenous agonist for PPAR-γ, 15d-PGJ_2. The study described in **chapter 9** was driven by the hypothesis that probiotics, which had already shown beneficial effects in stress-induced barrier dysfunction, modulate mast cell release resulting in a barrier protective effect. As expected, stress induced a vast increase in mucosal permeability to *E. coli* as well as an increase in bacterial translocation to mesenteric lymph nodes in a rat model of chronic stress. Pretreatment with probiotics ameliorated stress-induced barrier dysfunction and mucosal inflammation. Conversely, these beneficial effects of probiotics could not be reproduced in mast cell deficient animals. Furthermore, administration of probiotics resulted in reduced levels of rat mast cell protease (RMCP) II, suggesting that probiotics inhibited and or modulated degranulation of mast cells. In addition, mucosal 15d-PGJ_2 levels were elevated after probiotic treatment. The barrier protective effects of probiotics were abrogated in animals injected with a specific PPAR-γ antagonist, which suggests involvement of PPAR-γ. Taken together, the barrier protective effects of probiotics in stress-induced barrier dysfunction involve a mast cell and PPAR-γ dependent mechanism which is putatively mediated through mast cell-derived 15d-PGJ_2. Based on these data it is, however, impossible to determine whether these effects are solely dependent on PPAR-γ activation by mast cell derived 15d-PGJ_2 or partly mediated through a direct probiotic-induced activation of PPAR-γ within epithelial cells. Therefore, an *in vitro* experiment was designed to shed more light on mechanisms by which probiotics modulate mast cells and exert their protective effects. This model, as described in **chapter 10,** is the first *in vitro* model for barrier failure induced by CRH-activated mast cells and thereby resembling stress-induced barrier dysfunction, reported in literature. These *in vitro* experiments confirmed the hypothesis that probiotics modulate mast cell mediator profiles by demonstrating reduced release of TNF-α and increased release of 15d-PGJ_2. These effects could not be reproduced when mast cells were exposed to dead probiotic bacteria. However, treatment with filter-sterilized probiotic supernatant had similar modulating effects on mast cells, suggesting a role for soluble factors produced by probiotics. Yet again, the protective effects on epithelial barrier function of probiotics were largely dependent on mast cells and PPAR-γ. However, the finding that in the absence of mast cells probiotics still exerted barrier protective effects suggests that direct effects of probiotics on epithelial cells are also involved. This is putatively mediated by direct probiotic-epithelial cell contact as soluble factors alone did not show any protective effects in the absence of mast cells. Overall, probiotics and their soluble factors modulate mast cell function to release more 15d-PGJ_2 upon activation by CRH, resulting in PPAR-γ activation in epithelial cells and maintenance of mucosal barrier function despite stress-induced mast cell activation.

Limiting mucosal inflammation by activation of PPAR-γ induced by probiotics is especially interesting in the context of inflammatory bowel diseases as PPAR-γ activation is known to be reduced in colitis ulcerosa. Therefore, this novel mechanism of action of probiotics may facilitate development of treatment strategies to prevent stress-induced gastrointestinal symptoms and reduce the number of relapses in inflammatory bowel disease.

CONCLUSION

This thesis provides evidence that probiotics, administered before an illness occurs and thus under steady-state conditions, are able to maintain mucosal barrier function and limit disease severity once the illness occurs, putatively via enhancement of endogenous defense systems including anti-oxidative effects and activation of anti-inflammatory mediators like PPAR-γ. Yet before this mechanism of action can safely be employed to prevent disease, the exact nature of the probiotic mediators that exert these effects warrants further investigation. Furthermore, it should be determined whether these mediators indeed cause adverse effects in severely unbalanced systems and consequently need to be avoided in critically ill patients. However, a thorough understanding of how commensal, probiotic and pathogenic microorganisms interact with one another and their mammalian host will not only facilitate development of treatment strategies but will also allow us to fully appreciated the wonderful complexity of that small scale universe that is our microbiota and that determines so much of who we are.

SAMENVATTING

Probiotica zijn lang omschreven als 'goede bacteriën' of te wel 'bacteriën met gunstige effecten op de gezondheid'. Maar eigenlijk zou men deze definitie van probiotica moeten aanpassen. Omdat in 2008 behandeling met probiotica tot een hogere sterfte leidde bij patiënten met ernstig acute pancreatitis, is het op zijn plaats om de definitie te veranderen in 'bacteriën met gunstige effecten op de gezondheid, indien gegeven aan de juiste patiënt en op het goede tijdstip'.

Probiotica kunnen op verschillende manieren gunstige effecten hebben op de gezondheid. Dankzij jarenlang onderzoek naar de werking van probiotica zijn veel van deze mechanismen wetenschappelijk aangetoond. Het idee dat probiotica verdedigingssystemen van het lichaam versterken, doordat ze door het lichaam gezien worden als een kleine, schadelijke prikkel, is echter relatief nieuw en nog maar weinig onderzocht. Probiotica zouden op deze manier het lichaam kunnen voorbereiden op eventuele ziekte in de toekomst. Dit proefschrift is gebaseerd op de volgende hypothese: 'de gunstige effecten van probiotica zijn deels het gevolg van het stimuleren van de verdedigingssystemen.'

NO GUTS NO GLORY

We weten eigenlijk nog maar weinig van de complexe functie van onze darmbacteriën en in welke mate ze bijdragen aan onze gezondheid. Het is duidelijk dat na miljoenen jaren evolutie er een belangrijke symbiotische band is ontstaan tussen de gastheer en de bacteriën in zijn darm. **Hoofdstuk 2** geeft een overzicht van de nadelige effecten van stress op deze symbiose. Al vele jaren wisten patiënten dat stress een belangrijke rol speelt in het verloop van maag- en darmziekten, maar artsen waren daar minder van overtuigd. Intussen laat wetenschappelijk onderzoek zien dat stress inderdaad een aantoonbaar nadelige invloed heeft op bacteriën in het maagdarmkanaal en de barrièrefunctie van de darm. Onder stressvolle omstandigheden verandert de samenstelling van darmbacteriën doordat het aantal melkzuurbacteriën (lactobacillen en bifidobacteriën) afneemt terwijl gevaarlijke, pathogene bacteriën juist harder groeien. Daarnaast hechten juist deze slechte bacteriën zich beter aan de darmwand en kunnen soms de darmwand binnendringen.

Daarnaast wordt in **hoofdstuk 2** een mogelijke rol voor probiotica bij het voorkomen van deze negatieve gevolgen van stress beschreven. Probiotica hebben namelijk veel eigenschappen die deze negatieve effecten van stress kunnen tegengaan. Dit zijn onder andere: 1) het uitscheiden van stoffen die de groei van andere bacteriën remmen of bacteriën zelfs doden, 2) het verdringen van andere bacteriën door efficiënter gebruik te maken van de aanwezige voedingsstoffen, 3) het versterken van de darmbarrière door het beschermen van de structuren die epitheelcellen bij elkaar houden (tight junctions), 4) het verhinderen van apoptose (geprogrammeerde celdood) van epitheelcellen, en 5) het reguleren van de immuunreactie door bijvoorbeeld de activering van de nuclear factor (NF)-κB te beïnvloeden.

Epitheelcellen bekleden de binnenkant van de darmwand en beschermen het lichaam tegen bacteriën uit de darm. Probiotica hebben effect door direct contact te maken met deze darmcellen van de gastheer of door 'signaalstoffen' te produceren

in de darm. Butyraat is een voorbeeld van een 'signaalstof' die door probiotica kan worden geproduceerd. Het ontstaat bij vertering van koolhydraten en levert energie voor epitheelcellen in de dikke darm.

Het in **hoofdstuk 3** beschreven onderzoek is gebaseerd op de hypothese dat butyraat bijdraagt aan de barrièrefunctie van de dikke darm. *In vitro* werden epitheelcellen blootgesteld aan dinitrophenol waardoor de cel zijn belangrijkste bron van energie kwijt raakt. Wanneer vervolgens aan het model bacteriën (*E. coli*) werden toegevoegd, bleek de barrièrefunctie van het epitheel verstoord. Met als gevolg dat bacteriën zich een weg wisten te banen dwars door de epitheellaag heen. Butyraat herstelde de barrièrefunctie van het epitheel en hield bacteriën buiten de deur. Om verder onderzoek te doen naar de manier waarop butyraat deze beschermende effecten sorteert, werd er gekeken naar de route die bacteriën nemen om de epitheellaag te passeren. Bacteriën kunnen op twee manieren deze laag passeren: tussen de cellen door (paracellulair) of dwars door de cellen heen (transcellulair). Het feit dat butyraat geen effect had op paracellulaire passage suggereert dat bacteriën een transcellulaire route kiezen. Deze route kan beïnvloed worden door butyraat.

Naast een tekort aan energie kan ook verhoogde activiteit van het immuunsysteem resulteren in een verminderde barrièrefunctie van epitheel. Het bleek dat een toename in activiteit van het immuunsysteem (actief NF-κB) samenhangt met een toename van bacteriële passage door de epitheellaag heen. Door behandeling met butyraat kon de verhoogde activiteit van het immuunsysteem geremd worden, wat een gunstig effect zou kunnen hebben bij patiënten met inflammatoire darmziekten.

Deze nieuwe bevindingen onderstrepen het belang van probiotica en 'signaalstoffen' die ze produceren voor het behouden van de barrièrefunctie van epitheel. Om in de kliniek de beschermende effecten van butyraat te kunnen benutten, zou voeding die de groei van butyraat producerende bacteriën stimuleert (bv. havermout) gebruikt kunnen worden bij het voorkomen van inflammatoire darmziekten.

Ook probiotica zelf zouden de afbraak van de darmbarrière kunnen voorkomen. In **hoofdstuk 4** wordt een studie beschreven naar effecten van probiotica op de verspreiding en de groei van bacteriën tijdens ernstige ziekte. Wanneer de darmbarrière beschadigd raakt, kan dat desastreuze consequenties hebben. In het bijzonder wanneer dat gebeurt ten gevolge van ernstige ziekte zoals tijdens ernstige vormen van acute pancreatitis. Bacteriën zien dan de kans om vanuit de darm het totaal verzwakte lichaam binnen te dringen en zich te verspreiden naar andere organen. Dit kan ernstige gevolgen hebben, zoals multi-orgaan falen. Hoe deze kritieke situatie precies ontstaat, is lastig te bepalen omdat verschillende factoren een rol spelen. De toegenomen groei van gevaarlijke bacteriën, het gebrek aan doorbloeding van het darmslijmvlies en immuunreacties in de darm spelen allemaal een rol in de afbraak van de darmbarrière. Voorbehandeling met probiotica verminderde de groei van gevaarlijke bacteriën in een rattenmodel voor ernstige acute pancreatitis. Daarnaast nam de verspreiding van deze bacteriën vanuit de darm naar andere organen zoals lever en milt af. Dat de relatieve afname van verspreiding van levende bacteriën (gemeten d.m.v. conventionele kweken) groter was dan de relatieve afname van dode en levende bacteriën samen (gemeten d.m.v. polymerase chain reaction van bacterieel DNA), suggereert dat voorbehandeling met probiotica leidt tot een versterkt vermogen van de gastheer om bacteriële indringers te doden.

WHAT DOES NOT KILL YOU MAKES YOU STRONGER

Dankzij een niet aflatende stroom van potentiële gevaren in onze omgeving is het menselijk lichaam door evolutie voorzien van behoorlijk geraffineerde verdedigingssystemen. Wij zijn het levende bewijs van 'what does not kill you makes you stronger'. Onze verdedigingssystemen worden juist gestimuleerd door bedreigende prikkels uit de omgeving. Op deze manier wordt de staat van paraatheid aangepast aan het niveau van bedreiging. Het versterken van onze verdedigingssystemen heeft als resultaat dat het lichaam beter bewapend is tegen een eventuele aanval van ziekte.

Anti-oxidatieve verdediging

Tijdens ernstige ziekte vermindert de doorbloeding van de darm waardoor er een zuurstoftekort ontstaat. Door dit tekort aan zuurstof ontstaat oxidatieve stress en komen er zuurstofradicalen vrij die de darmwand aanvallen. Gelukkig zijn darmcellen voorzien van een verdedigingssysteem om schadelijke gevolgen van zuurstofradicalen tegen te gaan. Glutathion is hierbij de belangrijkste beschermer omdat het zuurstofradicalen aan zich bindt en zo een functie heeft als antioxidant. Dit is een voorbeeld van een verdedigingssysteem dat wordt versterkt door prikkels vanuit de omgeving. De cel heeft namelijk het vermogen zichzelf te verdedigen door de productie van glutathion te laten toenemen. Dit gebeurt onder andere wanneer de cel een verhoogde concentratie van zuurstofradicalen waarneemt.

Uit het rattenmodel, beschreven in **hoofdstuk 4**, blijkt dat met probiotica behandelde dieren beter in staat zijn om verspreiding van bacteriën naar andere organen dan de darm te voorkomen. Dat deze dieren de bacteriën beter in de darm lijken te houden zou logischerwijs het gevolg kunnen zijn van een verbeterde barrièrefunctie. Dit was de reden om in **hoofdstuk 5** in hetzelfde model voor ernstige acute pancreatitis en met dezelfde probiotica de specifieke werking van de darmbarrière onder de loep te nemen. Om te beginnen leidde acute pancreatitis tot een forse schade aan de barrière. Dit werd gekenmerkt door sterfte van epitheelcellen en vernietiging van 'tight junctions' (eiwit structuren die epitheelcellen bij elkaar houden). Dat zuurstofradicalen hierin een rol speelden, bleek uit een toename van oxidatieve schade aan de darm. Voorbehandeling met probiotica verbeterde de barrièrefunctie en verminderde het lek in de darm voor bacteriën. Daarnaast werd er minder oxidatieve schade gemeten in de met probiotica behandelde dieren. Dit zou het gevolg kunnen zijn van een betere bescherming tegen zuurstofradicalen. Om de mate van bescherming tegen oxidatieve stress te bepalen werd de concentratie van glutathion gemeten. In met probiotica behandelde dieren was niet alleen de concentratie van glutathion verhoogd, ook was de productie van glutathion toegenomen. Dat deze cellen zich beter zijn gaan verdedigen tegen zuurstofradicalen, kan een indicatie zijn dat zij blootgesteld zijn aan een milde vorm van oxidatieve stress en als gevolg daarvan hun verdedigingssysteem hebben geactiveerd. Toch staan de hier boven beschreven bevindingen in schril contrast met de negatieve effecten van probiotica zoals gerapporteerd in 2008. Toen leidde behandeling met probiotica bij een aantal acute pancreatitis patiënten tot een ernstig zuurstoftekort in de darm. Deze bevindingen lijken tegengesteld aan de resultaten beschreven in **hoofdstuk 5**. Om inzicht te krijgen hoe probiotica in patienten tot zulke ernstige effecten en zelfs tot hogere sterfte hebben geleid, is het van belang probiotica te beschouwen als milde oxidatieve prikkel. De hypothese dat probiotica een milde vorm van oxidatieve

stress veroorzaken, zou namelijk kunnen verklaren dat probiotica bij ernstig zieke patiënten negatieve effecten kunnen hebben. Het darmpakket van deze patiënten heeft immers al te leiden onder de gevolgen van verminderde doorbloeding. Een poging om bij deze patiënten het verdedigingssysteem te activeren met probiotica leidt alleen maar tot nog meer oxidatieve schade. Terwijl probiotica, gegeven vóór het ontstaan van de ziekte, juist een milde oxidatieve prikkel kunnen zijn om het lichaam te beschermen tegen oxidatieve stress.

Voorbehandeling met probiotica verbeterde niet alleen de verdediging tegen oxidatieve stress in de darm, ook andere organen bleken beter beschermd. Zo was de schade die ontstaat aan de alvleesklier tijdens acute pancreatitis minder ernstig in dieren die waren voorbehandeld met probiotica. Daarnaast verliep de immuunreactie in de alvleesklier milder en was de energievoorraad beter op peil. Vooral dat laatste is van belang, omdat een tekort aan energie kan leiden tot sterfte van alvleesklierweefsel. Bovendien was na voorbehandeling met probiotica de productie van glutathion toegenomen in zowel de alvleesklier als in rode bloedcellen. Deze resultaten onderstrepen het belang van de verdediging tegen zuurstofradicalen (**hoofdstuk 6**).

Een ernstig zieke patiënt komt in een vicieuze cirkel wanneer bacteriën de kans zien door de darmbarrière heen te breken. Het eerste orgaan waar deze bacteriën in terecht komen is de lever. De lever reageert hierop met een immuunreactie die vaak zo heftig is dat de bloeddruk verder daalt. Hierdoor wordt de darm nog slechter doorbloed, waardoor de barrièrefunctie alleen maar verder afneemt. Voorbehandeling met probiotica leidde tot een vermindering van de door pancreatitis veroorzaakte oxidatieve schade aan de lever (**hoofdstuk 7**). Vooral van belang is dat voorbehandeling met probiotica leidde tot verminderde activering van NF-κB. Activering van NF-κB wakkert de immuunreactie namelijk verder aan. Aangezien in de lever de grootste verzameling van immuuncellen huist, kan deze reactie nogal heftig verlopen. Activering van NF-κB is dan ook in eerdere studies in verband gebracht met multi-orgaan falen.

Therapie gericht op het versterken van de darmbarrière zou het verloop van ernstige ziekte kunnen verbeteren. Wel moet daarbij opgemerkt worden dat probiotica voor dit doel bij ernstig zieke patiënten geen goede keuze is. Om op een veilige manier van de beschermende effecten van probiotica gebruik te kunnen maken, lijkt een preventieve toepassing de beste keuze. Zo zou een dieet, aangevuld met probiotica, een onderdeel kunnen zijn van de preoperatieve voorbereiding van een patiënt. Vooral tijdens grote buikoperaties ontstaat er oxidatieve stress in de darmen en daarom zou voorbehandeling met probiotica de patiënt kunnen beschermen.

Anti-inflammatoire verdediging

Ons immuunsysteem heeft de neiging om sterker te reageren dan nodig is, omdat een onvoldoende reactie fatale gevolgen kan hebben. Dat we daarmee ook ons eigen lichaam beschadigen, nemen we op de koop toe. De mens is dankzij miljoenen jaren van evolutie uitgerust met prachtige mechanismen om de te sterke immuunreactie te nuanceren. Dit is van belang om de schade aan het lichaam binnen de perken te houden. Een belangrijke rol hierin is weggelegd voor de peroxisome-proliferator-activated receptor (PPAR)-γ. Activering van deze receptor kan de immuunreactie afremmen. Dendritische cellen die zich in het slijmvlies van de darm

bevinden, zijn de regisseurs van de immuunreactie in de darm. Door de uitvoerende cellen van de immuunreactie (T-cellen) te instrueren, bepalen zij in grote mate het verloop van de reactie. Dit werkt als volgt: na contact met bacteriën plaatsen dendritische cellen signaalstoffen (cytokines) op de buitenkant van hun celwand. De samenstelling van deze cytokines bepaalt het uiteindelijke gedrag van de T-cellen. Twee cytokines zijn daarbij van bijzonder belang: interleukine (IL)-12, dat bijdraagt aan een pro-inflammatoire, heftiger immuunreactie en IL-10, dat de T-cellen meer richting een milder type immuunreactie dwingt. Hoe dendritische cellen precies bepalen welk signaal ze geven is niet helemaal bekend, maar PPAR-γ lijkt daar een rol in te spelen.

Hoewel er steeds meer bekend is over de rol die dendritische cellen spelen in ontstekingsziekten van de darm, is er maar weinig bekend over hun rol tijdens ernstige acute pancreatitis. En dat terwijl juist bij deze ziekte de immuunreactie in de darm een bepalende factor is. **Hoofdstuk 8** beschrijft het eerste onderzoek naar het gedrag van dendritische cellen in de darm tijdens acute pancreatitis. Resultaten in dit hoofdstuk wijzen erop dat tijdens acute pancreatitis dendritische cellen bacteriën opnemen en IL-12 naar buiten brengen. Dit laatste zou T-cellen aan kunnen zetten tot een heftige immuunreactie. Na voorbehandeling met probiotica plaatsten dendritische cellen minder IL-12 op hun cell wand. Bovendien nam de hoeveelheid IL-10 in het slijmvlies van de darm toe. Deze verandering in de balans tussen IL-10 en -12 draagt bij aan een mildere immuunreactie door T-cellen. PPAR-γ kan het gedrag van dendritische cellen beïnvloeden en zou dus de oorzaak kunnen zijn van deze veranderde balans. In met probiotica behandelde dieren bleek dat PPAR-γ versterkt aanwezig was in dendritische cellen. Voorbehandeling met probiotica zou dus dendritische cellen kunnen aanzetten tot het initiëren van een minder heftige immuunreactie, mogelijkerwijs door een toename van PPAR-γ.

De activering van PPAR-γ speelt ook een belangrijke rol in het beperken van de ontsteking in de darm die kan ontstaan als gevolg van stress. Tijdens een stressvolle periode activeert het vrijgekomen stresshormoon bepaalde immuuncellen die aangeduid worden als mestcellen. Deze mestcellen zijn berucht om hun vermogen de barrière te beschadigen door het uitstoten van hun pro-inflammatoire inhoud. Door het vrijkomen van deze inhoud ontstaat er een ontstekingsreactie in de darm, waarbij de darmbarrière beschadigt wordt. Mestcellen zouden echter ook een beschermend effect op de barrière kunnen hebben. Het zijn namelijk de hofleveranciers van 15d-PGJ_2. Wanneer 15d-PGJ_2 vrijkomt kan het PPAR-γ activeren, de ontsteking remmen en zo de barrière beschermen. **Hoofdstuk 9** is gebaseerd op de hypothese dat probiotica de effecten van stress op de darmbarrière kunnen verminderen door mestcellen zo te beïnvloeden dat ze minder schadelijke inhoud en meer beschermende inhoud vrijgeven. Niet geheel onverwacht leidde chronische stress bij ratten tot een verminderde barrièrefunctie en konden bacteriën de darmwand passeren. Voorbehandeling met probiotica ondersteunde de barrièrefunctie en verminderde de ontstekingsverschijnselen in de darm. Probiotica hadden echter alleen effect in dieren met een normaal aantal mestcellen. In mestcel deficiënte dieren daarentegen, konden deze effecten van probiotica niet worden gereproduceerd.

In lijn met de hypothese dat probiotica de samenstelling van de vrijgekomen stoffen vanuit mestcellen veranderen, werd er in de met probiotica behandelde ratten verhoogde concentraties 15d-PGJ_2 gemeten in darmweefsel. Dat PPAR-γ

een rol speelt in dit beschermingsmechanisme, bleek uit het feit dat de protectieve effecten van probiotica volledig verdwenen wanneer de dieren geinjecteerd werden met een specifieke blokker voor PPAR-γ. Samenvattend spelen bij stress mestcellen en PPAR-γ een belangrijke rol in de beschermende effecten van probiotica, waarschijnlijk door een toename van het door mestcellen vrijgegeven 15d-PGJ_2. Deze resultaten laten echter niet zien of de effecten van probiotica volledig afhankelijk zijn van PPAR-γ, dat geactiveerd wordt door het door mestcellen geproduceerde 15d-PGJ_2, of dat een eventueel direct effect van probiotica op PPAR-γ in epitheelcellen ook een rol speelt. En daarom werd er een *in vitro* experiment ontworpen om te verhelderen hoe probiotica mestcellen beinvloeden en beschermende effecten uitoefenen. In dit model, dat beschreven wordt in **hoofdstuk 10**, worden mestcellen en epitheelcellen samen gekweekt. Door het stresshormoon aan dit model toe te voegen, worden de mestcellen geactiveerd en kan er in detail bestudeerd worden welk effect dit heeft op de epitheelcellen. Deze *in vitro* experimenten bevestigden de hypothese dat probiotica de samenstelling van de vrijgekomen stoffen uit mestcellen kunnen veranderen. Dit bleek uit het verminderd vrijkomen van het pro-inflammatoire TNF-α (dat de darmbarrière kan beschadigen) en een toegenomen afgifte van15d-PGJ_2 (dat een beschermend effect heeft). Interessant was dat niet *per se* de levende probiotica nodig waren om dit effect te bereiken. Ook wanneer de probiotica met behulp van de filter uit het medium werden gehaald en er dus alleen de oplosbare stoffen die probiotica produceren werden toegevoegd, werden dezelfde effecten op mestcellen gezien. Dit suggereert dat er een rol is voor door probiotica geproduceerde stoffen in dit beschermingsmechanisme. Ook in dit model was de bescherming van de epitheellaag door probiotica voor een groot deel afhankelijk van de aanwezigheid van mestcellen en PPAR-γ. Echter, dat probiotica ook zonder de aanwezigheid van mestcellen PPAR-γ in epitheelcellen kunnen activeren, wijst erop dat er mogelijk ook een direct effect van probiotica op epitheelcellen meespeelt. Aangezien door probiotica geproduceerde stoffen PPAR-γ niet konden activeren, lijkt direct cel-cel contact tussen probiotica en epitheelcellen van belang voor dit directe effect. Kortom, probiotica en de stoffen die door hen geproduceerd worden, beïnvloeden mestcellen met als gevolg dat deze meer 15d-PGJ_2 afgeven na activering door stress. Dit resulteert in een versterkte activering van PPAR-γ in epitheelcellen, wat leidt tot een betere bescherming van de darmbarrière tijdens stress.

Dat probiotica PPAR-γ activeren en op die manier ontstekingsverschijnselen in de darm kunnen remmen, is vooral interessant in het kader van inflammatoire darmziekten. Bij deze patiënten wordt PPAR-γ namelijk in mindere mate geactiveerd. Daarnaast zou dit nog niet eerder beschreven effect van probiotica bij kunnen dragen aan de ontwikkeling van functionele voeding die maag-darmklachten bij stress voorkomt.

CONCLUSIE

De experimenten beschreven in dit proefschrift laten zien dat probiotica, wanneer gegeven voorafgaand aan het ontstaan van ziekte, de darmbarrière kunnen ondersteunen en de ernst van de ziekte doen verminderen wanneer deze intreedt. Vermoedelijk, door het stimuleren van de verdedigingssystemen van het lichaam, waaronder de anti-oxidatieve verdediging en het activeren van een signaal om de

ontstekingsreactie af te remmen, zoals PPAR-γ. Echter, voordat deze effecten van probiotica veilig kunnen worden ingezet om ziekte te voorkomen, moet de exacte manier waarop probiotica deze effecten sorteren beter in kaart worden gebracht. Daarnaast zou gekeken moeten worden of probiotica via dit mechanisme inderdaad ook negatieve effecten kunnen hebben in systemen waarin de balans zo kritisch is dat de kleinste prikkel tot ernstige gevolgen kan leiden. Dit om te bepalen of probiotica terecht vermeden moeten worden in ernstig zieke patiënten. Hoe dan ook, alleen door meer inzicht te krijgen in de samenwerking tussen commensalen, probiotische en pathogene bacteriën en hoe zij hun gastheer beïnvloeden, kunnen we dat kleine maar zeer complexe universum dat in onze darmen huist op waarde schatten. Alleen zo zijn wij beter in staat te begrijpen wie we eigenlijk zijn.

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ABBREVIATIONS

15d-PGJ_2	15-deoxy-Δ prostaglandin J_2
ACTH	adrenocorticotropic hormone
ALT	alanine aminotransferase
ANOVA	analysis of variance
AP	acute pancreatitis
AP-1	activating protein-1
AST	aspartate aminotransferase
ATP	adenosine triphosphate
CFU	colony forming units
CRH	corticotropin-releasing hormone
DAPI	4′,6-diamidineo-2-phenylindole
DC	dendritic cell
DNP	dinitrophenol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERCP	endoscopic retrograde cholangiopancreatography
FAE	follicle-associated epithelium
GALT	gut-associated lymphoid tissue
GC	glutamylcysteine
GCL	glutamate cysteine ligase
GCLc	glutamate cysteine ligase catalytic subunit
GCLm	glutamate cysteine ligase modifier subunit
GFP	green fluorescent protein
GPC	gram-positive cocci
GPR	gram-positive rods
GRAS	generally regarded as safe
GSH	glutathione
GSSG	oxidized glutathione
H&E	hematoxilin and eosin
HIF	hypoxia-inducible factor
HPA	hypothalamic pituitary adrenal
HRP	horseradish peroxidase
Hsp	heat shock protein
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
ICE	IL-1β converting enzyme
IFABP	intestinal fatty acid binding protein
IFN-γ	interferon gamma
Ig	immunoglobulin
IKK	inhibitor κB complex
IL	interleukin
Isc	short circuit current
IκB	inhibitor κB
LAB	lactic acid bacteria

LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAMP	microbe-associated molecular patterns
MAPK	mitogen-activated protein kinases
M-cells	microfold cells
MDA	malondialdehyde
MLN	mesenteric lymph nodes
MODS	multiple organ dysfunction syndrome
MOF	multi organ failure
MPO	myeloperoxidase
MRS	deMan Rogosa Sharpe
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NF-κB	nuclear factor-κB
OCT	optimum cutting temperature
OD	optical density
OMG	O-methyl-D-glucopyranose
PB	probiotics
PCR	polymerase chain reaction
PFA	paraformaldehyde
PG	prostaglandin
PPAR	peroxisome proliferator-activated receptor
RBC	red blood cell
RBL	rat basophilic leukemia
RMCP	rat mast cell proteinase
ROS	reactive oxygen species
SCFA	short-chain fatty acid
SD	standard deviation
SED	sub-epithelial dome
SEM	standard error of the mean
SIRS	systemic inflammatory response syndrome
SS	sham stress
St	standard diet
STAT	signal transducer and activator of transcription
TEM	transmission electron microscopy
TER	transepithelial resistance
Th	T-helper
TJP	tight junction protein
TJ	tight junction
TLR	toll-like receptor
TNF	tumor necrosis factor
TUNEL	terminal-deoxynucleotidyl-transferase-mediated-dUTP-nick-end-labeling
WAS	water avoidance stress
Ws/Ws	mast cell deficient rats
ZO	zonula occludens

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CURRICULUM VITAE

Femke Lutgendorff was born on August 18, 1981 in Hengelo (Ov.), the Netherlands. After graduating *cum laude* at the van Maerlant Lyceum Eindhoven in 2000, she started her medical training at the University of Utrecht. After being introduced to laboratory animal science at the Department of Experimental Surgery, University Medical Center, Utrecht (Prof. Dr. L.M.A. Akkermans) she acquired the microsurgical techniques to study experimental pancreatitis under supervision of Dr. L.P. van Minnen. Equipped with these skills, she moved to Sweden to study pancreatitis-induced mucosal barrier dysfunction at the Department of Clinical and Experimental Medicine, Linköping University (Prof. Dr. J.D. Söderholm) in 2005. This experimental work was awarded with the Young Investigators Award at the World Congress of the International Hepato-Pancreato-Biliary Association in 2006 in Edinburgh, Great Brittan.

Femke Lutgendorff obtained her Medical Degree in October 2006, and started the work, described in this thesis at the Linköping University. Her PhD education was a joined venture of the Linköping University (Prof. Dr. J.D. Söderholm) and the University Medical Center, Utrecht (Prof. Dr. L.M.A. Akkermans and Prof. Dr. H.G. Gooszen). To gain experience in basic science she performed experiments at the Department of Physiology and Biophysics, University of Calgary, Canada (Prof. Dr. D.M. McKay) in 2009. Work in this thesis has been awarded several prizes among which; 'Best oral presentation' at the Swedish Surgical Week, Örebro, Sweden, in 2006, 'Best oral presentation' Annual Science Day, Department of Surgery, Utrecht in 2007 and 'Award of excellence' at the World Congress of the International Hepato-Pancreato-Biliary Association, Mumbai, India in 2008.

In August 2009 she enrolled in Pancreas 2000, a European program which aims to create an international network of physicians interested in pancreatology and will start her surgical residency in January 2010 at the Onze Lieve Vrouwe Gasthuis in Amsterdam (Dr. M. F. Gerhards).

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