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Pharmaco-nutritional approaches to combat heat stress-induced intestinal barrier dysfunction

Farmaco-nutritionele benaderingen om dysfunctie van de darmbarrière veroorzaakt door hittestress te vermijden

(met een samenvatting in het Nederlands)

Proefschrift

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door

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Its your road, and yours alone!

Others may walk it with you, but no one can walk it for you.

Rumi *(Mawlana)*

Contents

Chapter 1

General introduction and outline of the thesis

Under current predictions of climate change, it is assumed that the intensity and frequency of heat stress (HS)-related disorders are increasing in humans and animals [1,2]. HS, a condition in which the thermoregulatory mechanisms in the body fail to compensate for the heat production, is induced by exposure to high ambient temperatures (environmental/classic HS) and/or prolonged excessive activities, such as strenuous exercise (exertional HS) [3].

One of the primary organs which is affected by HS is the gastrointestinal tract [4,5], since the thermoregulatory mechanism of the body shifts the splanchnic blood flow towards the peripheral circulation in order to facilitate heat dissipation. The outcome is a splanchnic ischemia followed by hypoxia in visceral organs, especially the intestines, causing tissue injury and ultimately leading to "leaky gut syndrome" [6]. The disruption of the intestinal integrity facilitates the penetration of toxic luminal substances into the blood circulation, resulting in severe inflammatory conditions.

The gastrointestinal mucosa forms a selectively permeable barrier, separating the body from the intestinal lumen, and hence the environment. It effectively allows nutrient absorption, while it limits the intrusion of xenobiotics, antigens, toxins and pathogens [7]. At the luminal side of the intestinal mucosal barrier, a mucus layer secreted by specialized intestinal epithelial goblet cells, prevents the direct contact of many macromolecules and microbes with the intestinal epithelium [8]. In addition, this mucus layer contains biologically active substances, like antimicrobial peptides, which are controlling and preventing the migration of harmful micro-organisms into the mucus layer.

The intestinal mucosal surface is lined by epithelial cells, which are arranged as a monolayer tightened by an apical junctional complex, which is composed of tight junction (TJ) and adherens junction (AJ) proteins. The complex network of TJs and AJs seals the paracellular space between adjacent cells, thus preventing the paracellular translocation of luminal antigens and bacteria into the blood circulation [7,9]. TJs are composed of transmembrane proteins, such as claudins and occludin, and intracellular scaffolding proteins, such as zonula occludens (ZO) proteins, which provide a link between the transmembrane proteins and the actin cytoskeleton [10]. Similarly, AJs consist of the transmembrane protein E-cadherin that forms strong, homotypic interactions with cytoplasmic proteins, such as α-catenin and β-catenin, which interact with the actin cytoskeleton of adjacent cells [7]. Over the past decade various *in vitro* and *in vivo* studies have highlighted the vulnerability of apical junctional complexes, especially TJs, to hyperthermia conditions [4,11–13].

Following HS, efficient adaptation and resilience mechanisms can control cellular homeostasis in the intestines. The heat shock response (HSR) is a defence mechanism, which enhances the synthesis of heat shock proteins (HSPs) that prevent the aggregation and misfolding of proteins, thereby conveying a certain degree of resilience of cells to temporary hyperthermia [14]. Interestingly, HSPs, especially HSP70, are known to stabilize junctional complexes, thus playing a key

role in preserving the intestinal integrity under HS conditions [12,15]. While the protective role of HSPs was initially ascribed as an adaptation of cells to an elevated body temperature, it soon turned out that the HSR is a common cellular defence mechanism, which is activated by different stressors, particularly by an abundance of reactive oxygen species (ROS) [14].

Although there is a direct link between ROS scavenging function and tolerance to HS, ROS can also play a key role in mediating important signal transduction events under stress conditions [16]. Elevated intracellular ROS concentrations activate the nuclear factor erythroid 2 related factor-2 (Nrf2) pathway, which facilitates the upregulation of endogenous antioxidants, such as glutathione and haem oxygenase-1 (HO-1) [17–19]. It is known that the HSR and Nrf2 pathways target overlapping genes, thus cooperating and compensating for each other in the prevention of tissue injury [20]. In the intestines, maintenance of the redox balance stabilizes the intestinal barrier function under stress conditions [21,22].

The identification of the key role of cellular oxidative stress and HSR pathway in the maintenance of the integrity of the intestines, not only under HS conditions, but also in allergic and chronic infectious and metabolic diseases, has stimulated the search for dietary components that would be able to counteract these adverse effects.

Aims and outline of the thesis

The main objectives of this thesis are:

- To broaden the current knowledge about the vulnerability of the intestinal epithelium to HS-induced injury.
- To characterize the gut-health promoting effects of functional nutritional supplements, by selecting compounds of different classes and to elucidate the molecular mechanisms by which they may regulate the HSR, cellular redox status, intestinal integrity and inflammatory reactions under HS conditions.

The outline of the thesis is as follows:

Chapter 1: Presents a general background and the aims and objectives of the research described in this thesis.

Chapter 2: Provides an in-depth review on cellular mechanisms involved in the resilience of cells to HS and introduces nutritional supplements having the potency to prevent the HS-induced intestinal injuries based on their ability to regulate the intestinal epithelial cell homeostasis.

Chapter 3: Presents the validation of an *in vitro* model, in which human intestinal epithelial cells (Caco-2 cells) grow as confluent monolayers in transwell inserts, thus resembling the intestinal lining cells, are exposed to different conditions of hyperthermia, which gradually evoke HS and the corresponding HSR. This model was also used to test the hypothesis that pre-incubation of impermeable epithelial

monolayers with galacto-oligosaccharides (GOS) may prevent the HS-induced disruption of intestinal epithelial integrity.

Chapter 4: Describes an *in vivo* experiment with broiler chickens exposed to HS. Stimulated by the promising results in Chapter 3, GOS was added to the diet of the broilers to assess whether its protective effect on barrier integrity could be confirmed *in vivo*. Parameters monitored at the end of the 5-days challenge period included intestinal integrity, HSR, immunological parameters and markers of oxidative stress.

Chapter 5: Presents additional analyses of intestinal tissue samples from the chicken experiment described in Chapter 4, focusing now on the expression of intestinal brush border membrane nutrient transporters and their potential involvement in the pathophysiology of intestinal damage, induced by HS.

Chapter 6: This chapter was designed to test the hypothesis that the conditional essential amino acid L-Arginine may protect the epithelial integrity by stabilizing the HS-induced reduction of inducible nitric oxide synthase (iNOS)-mediated nitric oxide synthesis and the expression of AJs under HS conditions.

Chapter 7: This chapter is devoted to further explore the recognized relation between the HSR, oxidative stress and epithelial integrity. It could be shown that α-lipoic acid is able to suppress the HS-induced oxidative stress response. To demonstrate the effect of α-lipoic acid on cell proliferation, the so-called wound-healing assay was included in the experimental design.

Chapter 8: In this chapter the findings of this thesis are discussed with a particular focus on the modulation of a HSR by nutritional supplements. Additionally, the clinical relevance of the described findings and suggestions for intervention strategies are pointed out in this chapter.

Chapter 9: This final chapter presents a brief summary of all chapters of the thesis.

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Chapter 2

Beyond heat stress: intestinal integrity disruption and mechanism-based intervention strategies

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Abstract

The current climate changes have increased the prevalence and intensity of both, environmental and exertional heat stress (HS) conditions. One of the initial consequences of HS is the impairment of the intestinal epithelial barrier integrity due to hyperthermia and hypoxia following blood repartition, which often results in a leaky gut followed by penetration and transfer of luminal antigens, endotoxins and pathogenic bacteria. Under extreme conditions, HS may culminate in the onset of "heat stroke", a potential lethal condition if remaining untreated. Previous investigations highlighted the HS-induced alterations of the gastrointestinal epithelium and the association of a leaky gut with cellular oxidative stress, disruption of intestinal integrity and the exaggeration in the production of pro-inflammatory cytokines. This review aims to summarize the *in vitro* and *in vivo* evidence related to a group of nutritional supplements, which may increase the resilience to HS-induced intestinal integrity disruption and aims to discuss their mechanisms of action in maintaining intestinal epithelial homeostasis.

Introduction

The gastrointestinal (GI) tract is the largest surface of the body that is in contact with the outside environment. The intestinal epithelium is regarded as a physical and biochemical barrier between the luminal commensal and pathogenic microbial communities and the mucosal immune system [1]. Dysfunction of this barrier is caused by various pathological, toxicological and physical stressors including heat stress (HS), leading to local or systemic inflammatory reactions. Severe intestinal epithelial damage is considered as a major factor involved in HS-associated mortality [2–6].

One of the early investigations by Bynum *et al.* highlighted the positive correlation between the heat-induced hyperpermeability of the GI tract and the mortality caused by endotoxemia [7]. Conversely, the prophylactic administration of anti-lipopolysaccharides (LPS) hyper-immune plasma to primates subjected to high temperatures provided protection against heat stroke [8]. More recent investigations further unravelled the susceptibility of different organs to high temperatures demonstrating that the observed multi-organ failure is induced by a combination of heat-induced cytotoxicity, coagulopathies, and a systemic inflammation that affects not only the GI tract, but also other key organs and tissues, including the central nervous system [9], the kidneys [10], the liver [11] and the muscle tissue [12]. These findings suggest that the systemic dysfunction under HS conditions is not only associated with a dysfunction of individual organs, but is also a cumulative response. For instance, the increase in core body temperature shifts the splanchnic blood flow to peripheral tissues in order to dissipate excess body heat, leading in turn to hypoxia in visceral tissues [2]. HS-induced hypoxic conditions in the intestines result in disturbance of the balance between the production of reactive oxygen species (ROS) and the antioxidant defence system, leading to epithelial damage and an inflammatory response [13] (Fig. 1).

Considering the clinical relevance of HS-induced cellular oxidative stress, disruption of intestinal integrity and the local and systemic inflammatory responses, the aim of this review is to introduce promising nutritional intervention strategies, which may increase HS tolerance and to discuss their mechanisms of action, which explain their beneficial effects in maintaining and supporting the intestinal homeostasis.

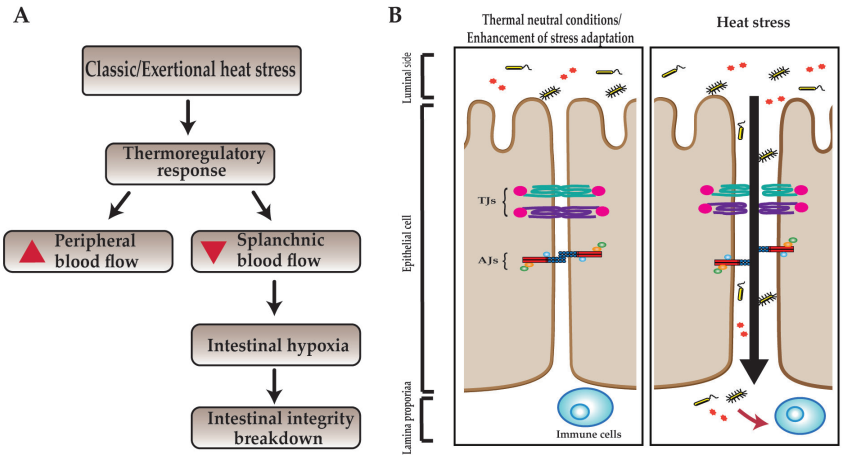

Figure 1. The sequence of events leading to heat stress (HS)-induced intestinal barrier damage. Hyperthermia induced by environmental or exertional HS stimulates the thermoregulatory mechanisms. A: In the whole body the thermoregulatory response shifts the splanchnic blood flow to the peripheral blood circulation, resulting in hypoxia in intestines and intestinal barrier dysfunction. B: At the cellular level, hyperthermia leads to disruption of intestinal epithelial integrity, mainly by affecting the tight junctions (TJs) and adherens junctions (AJs), which are responsible for sealing the paracellular space between adjacent cells. Damage to TJs and AJs facilitates the transfer of luminal LPS (lipopolysaccharides) (red heptagrams) and pathogens (yellow rod-shaped bodies) through the epithelial barrier into the lamina propria, harbouring numerous immune cells that are activated and contribute to the exaggeration of the inflammatory reactions, which may further worsen the intestinal damage.

Stress adaptation signalling pathways

The network of molecular structures underlying the cellular response to HS comprises multiple kinases, phosphatases, and transcription factors, each playing a crucial role in regulating the stress responses [14]. Under physiological conditions, there is an equilibrium between the components of this network. Upon exposure to stress stimuli and in order to protect the cell from damage, the cellular homeostasis changes. However, failure in this process would lead to unalterable signalling, leading to cell death [15].

HS and heat shock response

The heat shock response (HSR) was initially described as a specific molecular response of cells to adapt to elevated temperatures. Later, it was found that various environmental and pathophysiological stressors, which cause protein aggregation or misfolding, can induce a similar reaction [16]. HSR is regulated by the activation of a family of interacting transcription factors, the so called "heat shock factors (HSFs)", of which HSF1 is the best-characterized factor that is essential for the HSR

[16]. HSF1 contains a C-terminal and three N-terminal leucine zipper repeats and is located in the cytoplasm as a monomer. Under physiological conditions, it is bound to heat shock proteins (HSPs) and is inactive. Upon activation by cellular stressors, this complex of HSF1 and HSP dissociates. Liberation, trimerization and translocation of HSF1 into the nucleus activates heat shock elements (HSE) and initiates the transcription of more HSPs (Fig. 2A). HSPs fulfil an important role in binding to and protecting misfolded cellular proteins, a typical sign of HS. HSPs are classified into 5 different groups based on their molecular weight, structure and function, including families of small HSPs (molecular weight of 15–30 kDa), HSP60, HSP70, HSP90, and HSP110 [17]. The most stress-responsive marker is HSP70, which is usually expressed at low basal levels and increases in response to stressors to protect the cells from proteotoxic damages by binding to damaged proteins and aiding in the refolding of unfolded or misfolded proteins. Subsequently, HSPs inhibit apoptosis and even more important, the inflammatory response [14,16,18].

HSR and intestinal barrier integrity

Expression of HSPs, in particular HSP70, is associated with the stabilization of the actin cytoskeleton of intestinal cells preventing their aggregation under stress conditions [19]. Elevated levels of HSF1 and HSP70 are known to play a crucial role in increasing the expression of actin fibres in epithelial cells of the GI tract. Dokladny *et al.* showed that upon activation under HS conditions, HSF1 binds to the occludin promoter region mediating the upregulation of the expression and improving the participation of occludin in junctional complexes [20]. Exogenous HSP70 added to cell cultures is able to prevent the HS-induced alteration in permeability. Recently, it has been shown that upregulation of HSP70 in Caco-2 cells following exposure to gliadin is associated with a redistribution of HSP70 towards the cytoskeleton, thus strengthening the role of HSP70 in the maintenance of intestinal barrier function by direct interaction with tight junction (TJ) proteins [21]. We recently showed that one of the possible mechanisms by which the antioxidant α-lipoic acid (ALA) and the amino acid L-Arginine (L-Arg) preserve the intestinal integrity under HS conditions could be related to the enhancement of HSP70 expression [22]. The important role of HSP70 in stabilizing TJ proteins of endothelial cells and subsequently maintaining the integrity is also reported in the blood brain barrier [23]. A possible mechanism by which HSP70 attenuates the epithelial barrier dysfunction under stress conditions will be through preventing the activation of conventional protein kinase C (cPKC) thereby reducing the myosin light chain (MLC) protein phosphorylation of the actin cytoskeleton [24,25]. Another member of the HSP family, the Apg-2 (a member of the HSP110 subfamily), is known to interact directly with zonula occludens protein-1 (ZO-1) regulating the transcriptional activity of ZO-1-associated nucleic acid binding protein [26].

HSR and the immune system

In recent years, particular attention has been devoted to the immune-regulatory effects of HSPs, in particular HSP70. Translocation of xenobiotics and bacterial products, following intestinal epithelial damage under HS conditions may evoke an inflammatory response, which result in exaggeration of intestinal barrier dysfunction [24]. The anti-inflammatory properties of HSP70 have been studied extensively in chronic inflammatory disorders, such as inflammatory bowel disease (IBD) and celiac disease, as well as under conditions of hyperthermia [27–29]. Upregulation of HSP70 in response to HS is involved in the inhibition of pro-inflammatory cytokines expression [30]. It is speculated that HSPs block the production of pro-inflammatory cytokines by inhibiting the translocation of NF-κB to the nucleus [29]. Recently, van Eden *et al.* reviewed the effect of HSPs on expansion of anti-inflammatory regulatory T cells (Tregs) [31]. This specificity of HSPs is not limited to endogenous (self) HSPs, since administration of bacterial HSPs is also an effective strategy in treatment of inflammatory disorders [32–34]. Anti-inflammatory mechanisms of HSPs are beyond the scope of this review and a more complete description of these mechanisms can be observed in different reviews [35,36].

HS and oxidative stress response

Cell survival largely depends on the balance between ROS and cellular antioxidant mechanisms. The high reactivity of ROS can modify several cellular macromolecules, such as nucleic acids, proteins and lipids [14]. HS is a potent inducer of ROS production, which leads to tissue damages as soon as the cellular redox defence system consisting of glutathione (GSH), glutathione peroxidase, superoxide dismutase (SOD) and haem oxygenase 1 (HO-1) is exhausted [37]. It has been shown that hyperthermia, not only can provoke ROS production, but also is able to hamper the antioxidant defence system directly [38]. The expression of the antioxidant system is mainly regulated by nuclear factor erythroid 2 related factor 2 (Nrf2), which is repressed in the cytoplasm by the regulatory protein Kelch-like ECH-associated protein 1 (Keap1) under physiological conditions. Dissociation of Nrf2 from Keap-1 upon oxidative stress, leads to translocation of Nrf2 to the nucleus where it binds to the antioxidant response element (ARE) to induce the transcription of antioxidant proteins improving cell survival under stress conditions (Fig. 2B) [39,40].

The crosstalk between ROS and Nrf2 and/or Nuclear Factor-κB (NF-κB), which activates the inflammatory cascade is very complex and not yet fully elucidated [41].

Current evidence suggests that Nrf2 and HSF1 regulate overlapping target genes and that they may compensate for each other [42]. HO-1 (also known as HSP32) is considered as the most important Nrf2 target gene in facilitating NF-κB inhibition, which can be regulated by HSF1 as well. Additionally, it is demonstrated that exposure of HSF1 mutant cells to HS stimulates (although with delay) the

upregulation of HSP70 and HO-1, which is mediated by Nrf2 [43].

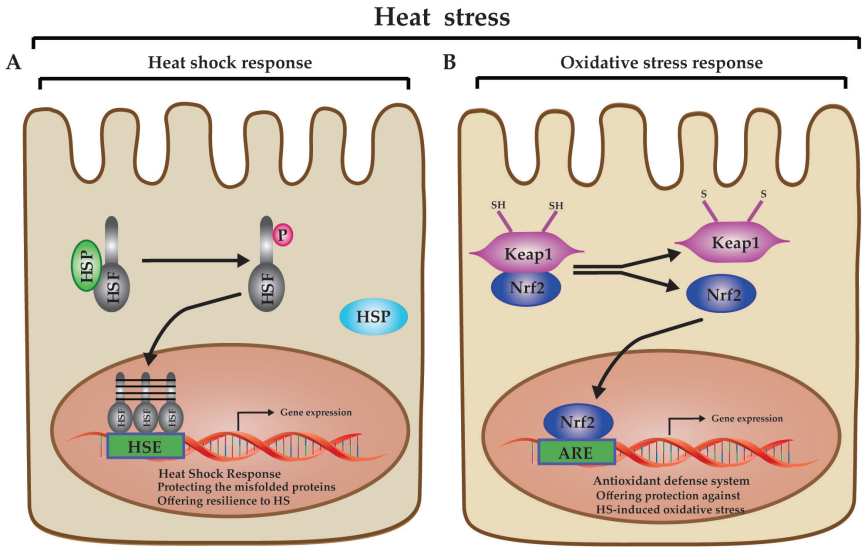

Figure 2. Schematic illustration of the heat stress (HS)-induced heat shock response and oxidative stress response. A: Cells under HS conditions activate the heat shock response pathway, which is initiated by translocation and trimerization of heat shock factor-1 (HSF1) into the nucleus, where it binds to the regulatory heat shock elements (HSE) in the promoter regions of heat shock protein (HSP) genes. B: Oxidative stress induced by HS, results in liberation of nuclear factor erythroid 2 related factor 2 (Nrf2) from Kelch-like ECH-associated protein 1 (Keap-1) and the translocation of Nrf2 into the nucleus where it binds to the antioxidant response element (ARE) in the promotor region of antioxidant target genes, driving their expression.

Intervention strategies against HS

Under HS conditions, change in the blood flow from visceral to peripheral circulation is identified as one of the major causes for ROS production, which leads to intestinal integrity disruption. It is shown that hyperthermia, not only can provoke ROS production, but also directly hampers the antioxidant defence system [38]. Numerous antioxidant substances including vitamins and plant polyphenols have been tested for their ability to reduce ROS-induced tissue damage [44–47]. In addition, selected amino acids and fatty acids are commonly recommended to mitigate disease conditions closely associated with cellular oxidative stress, as will be discussed below.

Independent from these direct effects on cells of the intestinal barrier system, the gut microbiota is also a common target of HS conditions [48,49]. Alterations in the composition of the gut microbiota together with the HS-induced impairment of the

barrier function, increase the likelihood of intestinal infections. In turn, pre- and probiotics have gained recent interest, as they are able to stabilize the intestinal microbiota under stress conditions.

Microbiota regulating substances

The gut microbiota, which comprises a vast array of microorganisms, has a key effect on regulation of host nutrition and metabolism, as well as on the stimulation of gut maturation, development, proliferation and immune homeostasis [48,50]. A variety of host conditions, including diet, host immune reactions, infections, and usage of antibiotics have the potential to influence the gut microbiota. Stress conditions, including HS, are known to induce alterations in the microbiota balance, which may result in the colonization of enteric pathogens [51], possibly leading to intestinal inflammatory responses [52]. Modulation of the gut microbiota composition is considered as an effective strategy to improve gut health and to protect the intestines against stress conditions. For example, ingestion of probiotics and prebiotics positively modifies the population of beneficial bacteria in the intestines, which is capable to stimulate the immune system and protect the intestinal integrity [53–55].

Probiotics

Probiotic bacteria are defined as "living microorganisms which exert health promoting benefits when administered in adequate amounts" [56]. A large range of bacteria are considered as probiotics, while the most common strains belong to *Lactobacillus* and *Bifidobacterium* spp. [57]. Probiotics are known to be protective against disorders, which influence the morphology and the immunological homeostasis in the GI tract of animals and humans. Furthermore, the beneficial effects of probiotics are related to the improvement of different components of the gut barrier system, including regulation of immune reactions, competitive displacement of pathogens and enhancement of intestinal epithelial cell integrity [58,59].

Intestinal barrier integrity

Different animal studies have shown that feed supplementation with probiotics alleviates the detrimental effects of HS on the microstructures of the small intestine, such as reduced villus height and villus area in broiler chickens [60,61]. An *ex vivo* study from Song *et al*. showed that treatment with a probiotic mixture (*Lactobacillus* and *Bifidobacterium*) would prevent the decreased Trans Epithelial Electrical Resistance (TEER) levels and increased paracellular permeability in the jejunal segment of HS-exposed chickens [61]. In this study, the beneficial effects of probiotics were associated with an increase in occludin and ZO-1 protein expression [61].

Feed supplementation with *Bacillus subtilis* is shown to improve the intestinal integrity development in chickens by increasing the expression of occludin,

claudin-2 and claudin-3 in the jejunum and the ileum [62]. Similarly, *Bacillus subtilis* is demonstrated to diminish the intestinal morphological changes and bacterial translocation as well as LPS penetration to the blood flow in rats exposed to HS [63]. Recently, a clinical study showed that 4 weeks of daily supplementation with a probiotic mixture maintains the intestinal integrity and reduces the penetration of LPS to the blood circulation in humans affected by intense exercise-induced HS [57]. Lamprecht *et al*. reported that supplementation with probiotics for 14 weeks reduced the marker zonulin, which indicates the enhanced gut permeability, in faeces of athletes [64].

Probiotics do not only interact with the bacterial populations in the intestines, but there is also a measurable interplay between microbiota and the host's defence systems. For example, probiotics seem to directly or indirectly modulate different signalling pathways that regulate the intestinal integrity, including Rho family GTPases, PKC, and mitogen-activated protein kinase (MAPK). It has been demonstrated that the protective effect of a *E. coli* Nissle probiotic on intestinal integrity of T84 cells challenged by enteropathogenic *E. coli*, is related to the stabilization of PKCζ and thereby preventing the phosphorylation and dissociation of ZO-2 from the TJ network [65]. In agreement with these findings, Hummel *et al.* showed that the epithelial barrier function in T84 cells (colonic adenocarcinoma epithelial cells) is enhanced by the Gram-positive probiotic lactobacilli via their effect on adherens junctions (AJs), including E-cadherin and β-catenin, by reducing the abundance of PKCδ in membrane junctional complexes [66]. *Lactobacillus brevis* is known to produce a bioactive molecule, polyphosphate, through activation of the integrin–p38 MAPK pathway, which leads to production of HSPs and prevention of oxidant-induced intestinal barrier disruption [67]. In addition, the protective effects of the probiotic strains *Streptococcus thermophiles* and *Lactobacillus acidophilus* on occludin phosphorylation in human intestinal epithelial cells challenged with enteroinvasive *E. coli*, can be inhibited by a Rho kinase inhibitor [68].

Intestinal Immunomodulation

Feed supplementation with the probiotic *Bacillus licheniformis* is shown to support the gut mucosal immunity in broiler chickens exposed to HS, by preventing the HS-induced increase in pro-inflammatory cytokines and the decrease in intraepithelial lymphocytes, the IgA secreting cells and mucin production [69]. Rajput *et al.* demonstrated that feed supplementation with *Bacillus subtilis* B10 stimulates the mucosal immunity development in broiler chickens by increasing IgA secretion and mRNA expression of the anti-inflammatory cytokine IL-10 [62]. Furthermore, clinical studies showed that dietary supplementation with a probiotic mixture increases the post-exercise plasma concentrations of IL-10 in exercise-induced HS [57].

The immune-regulatory properties of probiotics have been studied extensively in treatment of diseases affecting the intestinal mucosal immunity, such as IBD

[70,71]. It seems that the mechanism by which probiotics exert anti-inflammatory properties, is through inhibition of NF-κB [72]. Moreover, Jeon *et al.* speculated that probiotics are able to stimulate $CD103^+$ dendritic cells to produce IL-10 and IL-27 via the TLR-2/MyD88 pathway [73].
The activation of innate immunity by probiotics is mainly facilitated by microbe-associated molecular patterns, including cell wall polysaccharides and peptidoglycan [74], which interact with TLRs, C-type lectin receptors and nucleotide oligomerization domain-like receptors [75]. However, it shall be taken into account that as yet no single probiotic is found to be able to exert all the above mentioned effects. Therefore, current interest focuses on the use of combination products of pre- and probiotics, also denoted synbiotics, and other ingredients (such as antioxidants), to improve their beneficial effects in a broader range of clinical conditions.

Prebiotics
Dietary prebiotics are described as "selectively fermented ingredients that result in specific changes in the composition and/or activity of the GI microbiota, thus conferring benefit(s) upon host health" [76]. Human milk oligosaccharides (HMOs), a major component of colostrum, represent the first prebiotics in humans. Various attempts have been made to design alternative prebiotic oligosaccharides that mimic the health promoting effects of HMOs, including galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), which are widely used in infant formula [77]. These non-digestible oligosaccharides are not hydrolysed by digestive enzymes and reach the distal intestines, where they modify the autochthonous microbiota and exert a beneficial effect on the gut microbiota [78]. Selective stimulation of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, can induce immunomodulatory effects, enhance the intestinal integrity and preserve the intestinal micro-structures, which are indicated as the major effects of non-digestible oligosaccharides as functional foods [79,80]. It has been described that the gut microbiome targets different intracellular pathways via fermentation of non-digestible oligosaccharides and the subsequent production of short chain fatty acids (SCFAs), such as acetate, propionate or butyrate [81]. Butyrate is demonstrated to increase the antioxidant glutathione and to decrease ROS production when applied directly to the human colon [82,83], which would probably modulate the HS-induced intestinal damage by ROS [84].

Intestinal barrier integrity
In vivo investigations in chickens exposed to HS have reported that supplementation of the diet with mannan-oligosaccharides (MOS) and cello-oligosaccharides (COS) mitigated the heat-induced changes in intestinal morphology and intestinal barrier function [85,86]. Furthermore, MOS enhanced the intestinal integrity by increasing villus height, number of goblet cells and the populations of lactobacilli and

bifidobacteria, while at the same time reduced the *E. coli* load in the ceca of chickens [87]. Our recent investigation showed that dietary GOS supplementation would hamper the disruption of intestinal integrity by preventing the alterations in TJs and AJs in the jejunum of broiler chickens exposed to HS [88]. Zhong *et al.* indicated that GOS increase the number of intestinal bifidobacteria in rats and play a key role in prevention of intestinal integrity disruption by increasing the mRNA and protein expression of occludin [89].

Besides the effects on the gut microbiota, microbiota-independent effects and direct interactions of these oligosaccharides with different (immune) cells have raised more attention in recent years. Our *in vitro* investigations highlighted the effect of GOS on direct regulation of the intestinal integrity and junctional complexes to prevent the disruption of intestinal integrity induced by HS [90]. Another study from our group indicated that pre-treatment with GOS prevent the disruption of intestinal integrity by accelerating TJ reassembly and stabilizing the expression and cellular distribution of claudin-3 TJ protein in Caco-2 cells [91]. A recent study from Akbari *et al.* suggested that the microbiota-independent effect of non-digestible oligosaccharides on intestinal epithelial integrity depends on the oligosaccharide structure, size and concentration [92]. Although further research is needed to unravel the exact mechanism involved in the direct regulation of intestinal integrity by oligosaccharides, a recent *in vitro* study with T84 cells showed that COS promote TJ assembly by activating AMPK through calcium-sensing receptor-phospholipase C-IP_3 receptor channel-mediated calcium release [93].

Intestinal Immunomodulation

Our recent study in broiler chickens suggested that dietary GOS would prevent the HS-induced mRNA upregulation of IL-6 and IL-8 in the jejunum, however this effect could be related to the GOS-preserved maintenance of intestinal integrity [88]. In addition, we observed that GOS in the chicken diet prevented the HS-induced TLR-4 upregulation in the jejunum [88]. It can be speculated that disruption of intestinal integrity followed by translocation of luminal antigens and pathogens through the intestinal epithelium will induce exaggeration of TLR signalling, facilitate immune responses and eventually lead to the development of intestinal inflammation exacerbating intestinal injury [94–96]. Additionally, TLR-4 is described as a stress-related biosensor in initial injury responses [97] and may contribute to the intestinal barrier disruption, since it is demonstrated that TLR-4 knockout mice are protected from HS-induced intestinal hyper-permeability and microvascular endothelial barrier dysfunction [95,98].

In recent years, the immune-regulatory effects of prebiotics to prevent intestinal disorders, such as IBD and necrotizing enterocolitis, (food) allergy or intestinal damage related to mycotoxin exposure are extensively studied [54,99–103]. Akbari *et al.* showed that GOS suppress the mycotoxin-induced increase in CXCL8 in Caco-2 cells as well as the murine CXCL8 analogues (CXCL1 and CXCL2) in

the intestine [91]. Moreover, dietary GOS mitigated the inflammation-induced expression of the alarmin IL-33 in two different murine models [104]. Jeurink *et al.* reviewed the different mechanisms which can underlie the immune effects of dietary oligosaccharides [79].

ALA, a fatty acid with antioxidant properties

ALA is synthesized from octanoic acid in the mitochondria and is present in pro- and eukaryote cells, being identified as an important potent cellular antioxidant. Both reduced and oxidized forms of ALA retain the antioxidant potency by scavenging free radicals, exhibiting metal chelating activity and involvement in redox regeneration of other antioxidants (vitamins C and E) [105,106]. ALA is used as treatment for diverse pathologies associated with redox imbalances, including diabetes, ischemia-reperfusion injury and heavy metal poisoning. However, ALA may act as mild pro-oxidant by slightly increasing ROS concentrations to activate NRF2 and HSFs, and therefore increasing the resilience to stress conditions [106]. In addition to redox-regulating effects, ALA may enhance the gut integrity and exert anti-inflammatory properties [107–109]. We and others recently demonstrated that ALA is able to hamper the disruption of intestinal integrity and modulate the intestinal inflammation in models of HS, post-weaning diarrhoea and ulcerative colitis [107–109].

Intestinal barrier integrity

Although the gut preserving effects of ALA are not extensively studied under HS conditions, our recent investigations in Caco-2 cell monolayers exposed to HS showed that ALA can prevent the disruption of intestinal integrity by maintaining the protein expression and distribution of the AJ, E-cadherin. Furthermore, ALA also stimulates proliferation of intestinal epithelial monolayers and facilitates the reassembly of TJs [109].

ALA supplementation is known to preserve the intestinal integrity in oxidative and inflammatory disorders associated with intestinal damage [107,108,110,111]. ALA stimulates the recovery of the intestinal epithelial architecture by increasing the mRNA and protein expression of occludin and ZO-1 TJ proteins in a rat model for post-weaning diarrhoea. These findings are confirmed by *in vitro* studies with IEC-6 intestinal epithelial cells [108]. Ma *et al.* showed that ALA can mitigate the intestinal morphological damage by preventing the decrease in villus height and increase in crypt depth in glycinin-induced anaphylactic reactions in rats [111]. Additionally, ALA co- and post-treatment decrease the ulcerative colitis-induced gut permeability by maintaining the expression of occludin in mice [107]. It seems that these effects are at least in part, related to the regulation of the redox balance, since it is known that oxidative stress can induce a tyrosine-kinase-dependent dissociation of E-cadherin-β-catenin and occludin-ZO1 complexes, which leads to their cellular redistribution and a loss of barrier integrity [110]. Additionally,

the pro-oxidant activity of ALA stimulates the transcriptional activity of HSF1 to induce the expression of HSP70. HSP70 may be involved in the maintenance of barrier integrity through direct interaction with TJ proteins and stabilizing the junctional complexes [21].

Intestinal Immunomodulation

The anti-inflammatory effects ALA are closely related to its antioxidant properties. As mentioned before, activation of the NRF2 transcription factor by ALA results in the induction of HO-1, which exerts anti-inflammatory effects by degrading intracellular haem to free ion, carbon monoxide and biliverdin [112,113]. In the last decade, an increasing number of findings highlight the effect of ALA in the transcriptional regulation of genes associated with inflammatory pathways [106,114,115]. We recently showed that exposure of intestinal epithelial Caco-2 cells to HS will increase the cyclooxygenase-2 (COX-2) expression, which is attenuated by ALA pre-incubation [109]. Interestingly, inhibition of COX-2 by ALA, is speculated to be important in prevention of ulcerative colitis in rats [116]. Another investigation showed that ALA co- and post-treatment in mice with ulcerative colitis not only prevent the transcriptional activation of COX-2, but also significantly reduces various inflammatory markers, such as myeloperoxidase, IL-17, IL-6 and TNF-a in the colon [107].

These findings support the hypothesis that the anti-inflammatory and protective effects of ALA under stress conditions are mainly attributable to the inhibition of IKB/NF-κB phosphorylation, hence preventing the activation of NF-κB [115].

L-Arg, a non-essential amino acid

In the last decade, the importance of some amino acids have been broadened from nutritional-only to therapeutically-important agents, due to their ability to modify cell signalling and to modulate gut-associated disorders [117–119].

It is indicated that L-Arg supplementations can attenuate the adverse effects of heat stroke in *in vivo* models [120,121]. Therapeutic administration of L-Arg in mice and rats exposed to HS, is associated with a reduction of the adverse effects of multi-organ failure, such as circulatory shock and cerebral ischemia, leading to improved survival [120–122]. L-Arg supplementation is also involved in the maintenance of intestinal homeostasis. Sukhotnik *et al*. found that oral administration of L-Arg significantly enhances the intestinal recovery and accelerates the mucosal repair following ischemia-reperfusion injury in rats [123]. An *in vitro* investigation showed that L-Arg suppresses the apoptosis and cell death, induced by LPS in IPEC-1 cells [124]. However, it seems that the role of L-Arg in intestinal inflammation can be introduced as "double-edged sword", because supra-physiological concentrations of L-Arg (>10 mM) may inhibit the cell migration in intestinal wound edges and play a deleterious role in the pathogenesis of inflammation [125,126]. Similarly, other investigations have reported that supra-physiological concentrations of L-Arg

worsen the mucosal damage and gut barrier function after ischemia/reperfusion injury in rats [127]. However, L-Arg supplementation in a physiological range plays an important role in the metabolic synthesis pathways, like the polyamine and nitric oxide (NO) production, which are involved in multiple cellular signalling pathways in enterocytes, including intestinal protein synthesis, blood flow, healing processes and intestinal immunity [128,129].

It is known that HS can block the physiological NO production [130] and it has been reported that preventing the NO synthesis will significantly increase the body heating rate, reduce the heat dissipation and increase the intestinal epithelial permeability [131,132]. Therefore, basal NO levels can be considered as a key factor in the enhancement of resilience to stress conditions [133]. Available evidence shows that physiological NO production can enhance the tolerance to HS by reducing O_2 costs under extensive exercise [134,135].

Intestinal barrier integrity

New findings describe that dietary L-Arg supplementation plays an important role in attenuating the intestinal integrity disruption caused by exertional hyperthermia [131]. Our recent investigations showed that pre-treatment of Caco-2 cells with non-toxic L-Arg concentrations can prevent the disruption of intestinal integrity [22]. *In vivo* investigations have also confirmed the effect of L-Arg supplementation in improvement of intestinal integrity and preservation of TJs in experimental models of IBD and hypoxia [136,137]. Tanaka *et al.* demonstrated that L-Arg supplementation will increase the mucus production as well as fluid secretion and inhibit intestinal hyper-motility in rats [138]. Additionally, *in vitro* and *in vivo* studies have indicated that supplementation with L-Arg, prevents the bacterial translocation by reducing intestinal necrosis, increasing villus height, and attenuating gut mucosal injury [129,139].

Different mechanisms may be involved in the L-Arg-induced tolerance of intestinal epithelial cells to HS, including:

I) The NO synthesis pathway: L-Arg, as a precursor of NO production, stimulates the enzyme NO synthase (NOS) isoforms to facilitate the synthesis and bioavailability of NO [140]. The constitutive form of NOS (cNOS), which includes endothelial NOS (eNOS) and neuronal NOS (nNOS), generates relatively small amounts of NO, while inducible isoform of NOS (iNOS) produces a quantitatively larger amount of NO and is expressed in cells of the immune system as well as in intestinal epithelial cells [118,141,142].

Although the precise mechanisms through which NO protect the intestinal integrity is not fully understood, it is assumed that NO regulates the intestinal integrity by modulating intracellular signalling pathways related to protein tyrosine phosphorylation in epithelial cells [143]. Protein tyrosine phosphorylation of TJ and AJ proteins, which can be induced by diverse oxidation-related stimuli including HS, can be involved in barrier disruption under oxidative stress conditions [144,145].

Additionally, NO signalling plays a key role in intestinal re-epithelialization and maintenance of intestinal integrity following mucosal injury [137,146].
II) Mammalian target of rapamycin (mTOR) pathway: Maintaining the intestinal epithelial function by L-Arg can also be related to activation of the mTOR pathway [125,147]. L-Arg is known as an optimal amino acid that can induce the downstream mTOR pathway by phosphorylation and activation of the protein synthesis regulator p70 S6 kinase ($p70^{S6k}$) [148]. Activation of $p70^{S6k}$ by L-Arg will increase the protein synthesis, proliferation and migration in disease conditions that induce intestinal epithelial injury [129]. For instance, oral administration of L-Arg in a porcine model of enteritis, can augment the intestinal protein synthesis and attenuates intestinal permeability via mTOR signalling and $p70^{S6k}$ activation [147].
III) Arginase pathway: Metabolism of L-Arg via the arginase pathway results in the production of ornithine and polyamine, which promote intestinal epithelial repair and restitution processes [125]. It is speculated that polyamines are involved in the regulation of cell-cell interactions and E-cadherin expression, being critically important for the maintenance of intestinal epithelial integrity [149]. Additionally, polyamines are important stress-responsive molecules, which facilitate the activation of HSF1 to induce HSP expression [150,151].

Intestinal Immunomodulation

Available evidence indicate that NO synthesis from L-Arg and the subsequent production of intestinal secretory immunoglobulin A (sIgA), modulate the expression of Th1/Th2 cytokines and prevent the exaggerated inflammatory responses followed by intestinal damage [138,152]. Costa *et al.* speculated that one of the beneficial effects of L-Arg supplementation in preventing the intestinal inflammation in rats exposed to HS, may be mainly attributable to these immune-regulatory effects [131]. Coburn *et al.* demonstrated that L-Arg supplementation will markedly reduce the expression of pro-inflammatory cytokines in the colon of mice with an experimental sodium dextran sulphate-induced colitis [136].
It is suggested that the main mechanism of action by which L-Arg modulates the inflammatory responses, is the iNOS-induced NO production, which inhibits NF-κB [153], since inhibition of iNOS leads to the loss of all clinical benefits of L-Arg in the intestines [136].

Conclusions

HS is considered as an important environmental and exertional hazard that is of increasing public health concern. Intervention strategies that can prevent, control and reduce the pathologies (and even mortality) due to HS in humans and animals, are therefore gaining increasing attention. The disruption of intestinal integrity followed by a generalized inflammatory response, is a key event in human and animal pathologies under HS conditions. Therefore, an increasing number of studies focus on the understanding of the molecular mechanisms involved in HS-induced intestinal hypoxia, inflammation and intestinal barrier disruption with the aim to introduce efficient strategies to preserve the physiologic performance of gut epithelium.

Nutritional substances, which have the potency to preserve not only the cellular homeostasis by enhancing the non-specific cellular defence systems, but also maintain the intestinal integrity are considered as optimal feed/food supplements to protect animals and humans against the adverse effects of HS.

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Chapter 3

Galacto-oligosaccharides exert a protective effect against heat stress in a Caco-2 cell model

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Abstract

Thermal stress can evoke a stress response and enhance the synthesis of heat shock proteins, while gut barrier dysfunction is considered as an important adverse effect of thermal stress. Considering the previously described effects of galacto-oligosaccharides, nowadays mainly used in infant formulas, we hypothesized that galacto-oligosaccharides may protect the intestinal barrier against heat stress (HS). Human epithelial colorectal adenocarcinoma cells were pre-treated with galacto-oligosaccharides prior to thermal stress exposure (40-42°C) for 24h. Pre-treatment of galacto-oligosaccharides prevented the HS-induced upregulation of heat shock proteins and reduced the heat-induced stress response as observed by a decrease in haem oxygenase-1. Galacto-oligosaccharides partly prevented the heat-induced effects on monolayer integrity as measured by Trans Epithelial Electrical Resistance (TEER), paracellular permeability and E-cadherin expression. In addition to their prebiotic effect, galacto-oligosaccharides may have beneficial potency to protect the intestinal epithelial barrier against HS and may be an attractive dietary application for people who are at high risk of developing HS.

Introduction

The cellular stress response is a protective reaction of individual cells to potentially harmful internal and external stimuli. It is well established that exposure of cells to various stressors, including thermal stress, oxidative stress, or pathological conditions like ischemia, tissue damage, infection and inflammation [1,2] can evoke a stress response and enhance the synthesis heat shock proteins (HSPs), via activation of heat shock factors [3]. HSPs prevent stress-induced protein aggregation and misfolding, and promote their return to native conformations maintaining protein homeostasis [4]. They are classified into different groups based on their molecular weight, structure and function, including families of small HSPs (molecular weight of 15-30 kDa), HSP60, HSP70, HSP90 and HSP110 [5]. Although HSPs are generally considered to improve cellular recovery, imbalances in HSP70 and HSP90 levels can induce cell growth arrest and developmental defects [6]. An alteration in the expression of HSPs, but also thermoregulatory failure and dysregulation of the acute-phase response may contribute to the progression of HS into heat stroke. Heat stroke is a potentially fatal disorder characterized by multi-organ injury and an elevated core body temperature that rises above 40°C. Heat stroke may result from exposure to high environmental temperatures (classical heat stroke) or as a consequence of extensive exercise (exertional heat stroke) [7]. Heat-induced multi-organ injury may include varying degrees of central nervous system dysfunction, acute renal failure, liver failure, skeletal muscle injury and gut ischemia [8]. An important early symptom of thermal stress is a dysfunction of the intestinal barrier leading to increased intestinal permeability and as a consequence increased entrance of toxic luminal substances [9,10]. With the prospect of increasing global warming and increase in frequency and intensity of HS [11], it is important to investigate preventive measures that can alleviate adverse effects of exposure to high environmental temperatures. Food supplemented with non-digestible oligosaccharides, including galacto-oligosaccharides (GOS) are known to support the maintenance of the gut homeostasis, protect the intestinal barrier integrity and stimulate gut associated immunity [12,13]. Considering these effects of GOS on improving gut health, we hypothesized that dietary GOS might protect the epithelial barrier against the heat stress (HS)-induced effects on HSPs expression levels, on oxidative stress, and on the intestinal barrier integrity. In this study, an *in vitro* epithelial colorectal adenocarcinoma (Caco-2) cell culture model was used as a model to assess the effects of thermal stress on the expression of HSPs as well as on the intestinal barrier function and to investigate the potential protective effects of GOS. Results show that in this *in vitro* model, dietary GOS prevented heat-induced upregulation of HSPs and markers of oxidative stress. The HS-induced disruption of the intestinal barrier was mitigated by GOS especially by modulating epithelial-cadherin (E-cadherin) expression.

Materials and Methods

Galacto-oligosaccharides (GOS)

The commercial product Vivinal® GOS syrup (FrieslandCampina Domo, Borculo, The Netherlands) containing galacto-oligosaccharides with a degree of polymerisation (dp) of 2-8 was used. The final product contained approximately 59% (w/w) galacto-oligosaccharides, 21% (w/w) lactose, 19% (w/w) glucose and 1% (w/w) galactose on dry matter (dry matter of 75%) and dilutions [1% and 2.5% (w/v) GOS] were produced in complete cell culture medium. Before starting the experiments close to equimolar concentrations of 21% (w/v) lactose and 19% (w/v) glucose as present in the 2.5% GOS solution were tested in the Caco-2 cell assays and no effect on the HS-induced Trans Epithelial Electrical Resistance (TEER) decrease and increase in paracellular Lucifer Yellow (LY) flux was observed (data not shown).

Cell culture

Caco-2 cells were obtained from the American Type Tissue Collection (Code HTB-37) (Manassas, VA, USA, passages 5-19) and were grown as a monolayer in Dulbecco's modified Eagle's minimum essential medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 25 mM Hepes, 4.5 g/l glucose (Invitrogen corp., Carlsbad, CA, USA), 10% (v/v) inactivated Fetal Calf Serum (FCS) (Gibco), Glutamine (2 mM, Biocambrex, Verviers, Belgium), 1% (v/v) non-essential amino acids, Penicillin (100 U/ml) and Streptomycin (100 μg/ml) (Biocambrex) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Confluent cells (90%) were trypsinized using 0.05% trypsin and 0.54 mM ethylene-diamine-tetra-acetic acid (EDTA). All heat shock experiments were performed with Caco-2 cells seeded on 0.3 cm^2 high pore density polyethylene terephthalate membrane transwell inserts with 0.4 μm pores (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) placed in a 24-well plate. The Caco-2 cells were seeded at a density of 0.3×10^5 cells/transwell insert. All these transwell experiments were started after obtaining a confluent Caco-2 monolayer at day 17-19 of culturing with TEER values in the range of 400 $\Omega.cm^2$.

Induction of HS

The cells were cultured at temperatures of 37°C (normal temperature for cell culture), and alternatively at 40°C, 42°C for the induction of HS in a humidified atmosphere of 95% air and 5% CO_2 for 2, 4, 6, 8, 12 and 24h. Temperatures of 40°C and 42°C were selected to reflect temperatures potentially causing slight or harmful HS to the cells, respectively. Prior to heat exposure, Caco-2 cells were pre-treated with either cell culture medium (control) or medium supplemented with GOS (1 or 2.5%) for 24h.

Cell viability assay

Cytotoxicity induced by HS exposure (24h) and GOS (1% and 2.5%) was measured by the release of lactate dehydrogenase (LDH) in culture medium of the apical and basolateral compartment using the CytoTox 96 Non-Radioactive CytoToxicity Assay Kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions.

TEER measurement

The integrity of the Caco-2 monolayer was determined prior to and after HS exposure (2, 4, 6, 8, 12 and 24h) by measuring TEER levels using a Millicell-ERS voltohmeter (Millipore, Temecular, CA, USA). Average TEER values for untreated cell monolayers were in the range of 412±20 $\Omega.cm^2$. The results are expressed as a percentage of initial value.

Paracellular permeability assay

Paracellular permeability across the Caco-2 cell monolayer was determined by measuring the flux of LY (molecular mass of 0.457 kDa). The transport studies from the apical side to the basolateral side were performed with 20 μg/ml of LY (Sigma Chemical Co, St Luis, MO, USA) which was added to the apical compartment (300 μl) of the transwells, 4h prior to the end of HS exposure. Medium from the basolateral compartment was collected 4, 12 and 24h after exposure to HS. The amount of LY in the basolateral compartment was determined by measuring the fluorescence intensity using a fluorometer (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany) at excitation and emission wavelengths of 410 and 520 nm.

RNA extraction and quantitative RT-PCR (qRT-PCR)

The levels of mRNA expression of different target genes [HSP70, HSP90, claudin-1, -3 and -4, occludin, zona occludens protein-1 (ZO-1), E-cadherin and haem oxygenase-1 (HO-1)] were measured by qRT-PCR in Caco-2 cells pre-treated with or without GOS for 24h and exposed to HS for 6, 12 and 24h. Cells were harvested with RNA lysis buffer containing β-mercaptoethanol. Total RNA was isolated using spin columns based on manufacturer's instructions (Promega). RNA was reverse-transcribed to cDNA using iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA).

For qRT-PCR, the PCR reaction mixture was prepared and amplifications were performed using iQSYBR Green Supermix (Bio-Rad) according to manufacturer's instructions using the MyiQ single-colour real time PCR detection system (Bio-Rad) with the MyiQ System Software version 1.0.410 (Bio-Rad). Commercially manufactured sets of gene specific primers (Eurogentec, Seraing, Belgium) were used after confirmation of specificity and efficiency analysis by qRT-PCR with dilution series of pooled cDNA at a temperature gradient (55°C to 65°C) for primer annealing and subsequent melting curve analysis (Table S1). The mRNA quantity

was calculated relative to the expression of β-Actin reference gene.

Western blot analysis

Caco-2 cells pre-treated with or without GOS for 24h and exposed to HS for 12 and 24h were lysed using 50 μl RIPA lysis buffer (Thermo scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany) and total protein concentration was assessed by a BCA protein assay kit (Thermo scientific). Equal protein amounts of boiled samples were separated by electrophoresis (Criterion™ Gel, 4-20% Tris-HCL, Bio Rad, USA) and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad, Veenendaal, The Netherlands). Membranes were blocked with PBS containing 0.05% (v/v) Tween-20 (PBST) and 5% (w/v) milk proteins and incubated overnight at 4°C with antibodies for HSP70, HSP90 (1:1000, Enzo Life Sciences, Farmingdale, NY, USA), claudin-1, -3, -4, occludin, ZO-1 (1:1000, Invitrogen Corp.), E-cadherin (1:1000, eBioscience, San Diego, CA, USA) or caspase-9 (1:1000, Abcam, Cambridge, UK). After washing in PBST, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000, Dako, Glostrup, Denmark) for 2h at room temperature. Finally, blots were washed in PBST and incubated in commercial ECL reagents (Amersham Biosciences, Roosendaal, The Netherlands) and exposed to X-ray film (Thermo scientific). Membranes were probed with monoclonal rabbit anti-human β-Actin antibody as well (1:2000, Cell Signaling, Danvers, MA, USA) to evaluate equality of loading. Films were scanned on a GS710 calibrated imagine densitometer (Bio-Rad) and the optical density (OD) for the immune-reactive bands was quantified and expressed as relative protein expression (optical density normalized with β-Actin).

Immunofluorescence staining

Immunofluorescence staining was performed to determine cellular localization of HSP70, HSP90 and E-cadherin. Caco-2 cells were grown on inserts and treated as described above. The inserts with Caco-2 cells were fixed with 10% formalin and after washing with PBS, the cells were permeabilized with PBS containing 0.1% (v/v) Triton-X-100 for 5 minutes, followed by blocking with 5% serum in 1% (w/v) bovine serum albumin (BSA)/PBS for 30 minutes at room temperature. Thereafter, Caco-2 cells were incubated (2h at room temperature) with primary antibodies of HSP70 (1:50, Enzo Life Sciences), HSP90 (1:50, Abcam) and E-cadherin (1:50, BD biosciences) followed by incubation with Alexa-Fluor conjugated secondary antibodies (Invitrogen) for 1h at room temperature. Nuclear counterstaining was performed with Hoechst 33342 (1:2000, Invitrogen) and subsequently the inserts were washed and mounted with ProLong Gold anti-fade reagent (Invitrogen). HSPs and E-cadherin were visualized and images were taken using the Nikon Eclipse TE2000-U microscope equipped with a Nikon Digital Sight DS-U1 camera.

Statistical analysis

Analyses were performed by using GraphPad Prism (version 6.0) (GraphPad, San Diego, CA, USA). Experimental results are expressed as mean ± SEM and differences between groups are statistically determined by using One-way or Two-way analysis of variance (ANOVA), with Bonferroni post-hoc test. For single factor experiments one way analysis is performed, while for experiments comprised of two factors a two-way ANOVA is used. Results are considered statistically significant when $P < 0.05$. Changes in mRNA relative expression between groups are statistically determined when the expression ratio of two-fold or higher is observed [14].

Results

HS does not affect cell viability

To determine the effects of GOS as well as HS exposure without treatment on the survival of the Caco-2 cell monolayers, a LDH leakage assay was performed. The results indicated that neither GOS in the used test concentration nor HS at 40°C and 42°C for 24h did impair Caco-2 cell viability (Fig. S1).

HS upregulates the mRNA expression of HSPs and disrupts intestinal barrier integrity

Before investigating the effects of GOS, the effect of HS on HSP gene expression and intestinal barrier integrity was investigated at different time points to design an *in vitro* HS model. Exposure of Caco-2 cells to 40°C and 42°C induced an upregulation of mRNA expression levels of HSP70 and HSP90 after 6, 12 and 24h (Figs. 1A and 1B). A temperature-dependent effect could be clearly observed for both HSPs, and early stage responses (6 and 12h) showed higher HSP mRNA expression levels compared to the 24h HS response. The HSP70 mRNA levels were more pronounced after HS exposure compared to HSP90, since a 77-fold increase was achieved in the levels of HSP70 mRNA expression, whereas the mRNA expression levels for HSP90 was increased 11-fold after 6h HS exposure (Figs. 1A and 1B). Furthermore, changes in intestinal monolayer integrity were determined by TEER measurement and paracellular transport of LY (0.457 kDa) across the Caco-2 cell monolayer. Results indicated that after HS exposure (40°C and 42°C), TEER levels decreased temperature- and time-dependently during 24h compared to the control group of 37°C (Fig. 1C). In line with these results, HS exposure also induced a temperature-dependent increase in the translocation of LY from the apical to the basolateral side after 12 and 24h HS (Fig. 1D).

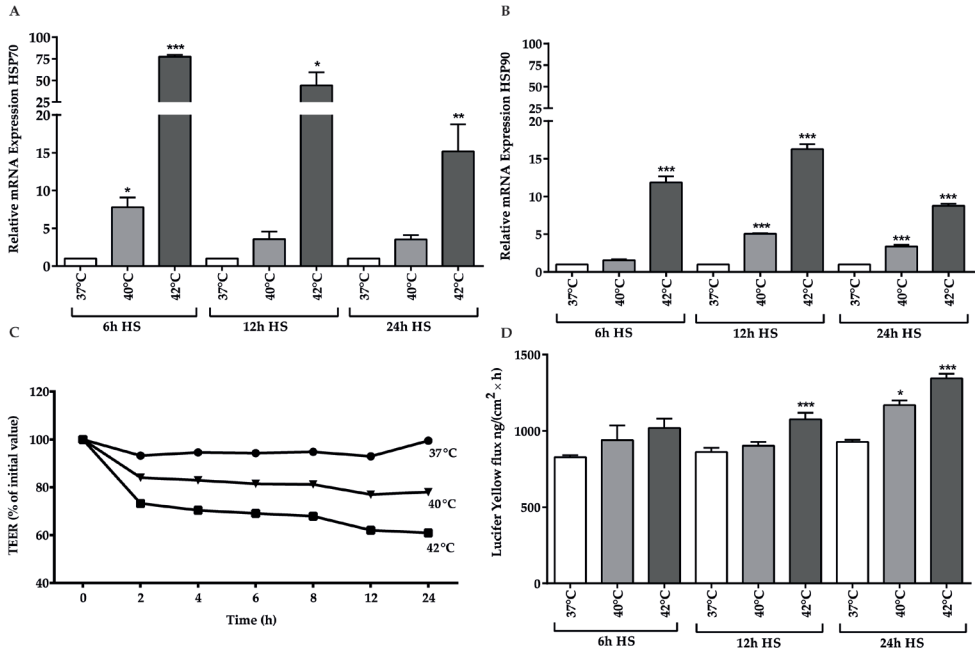

Figure 1. HS upregulates the HSPs mRNA expression and disrupts intestinal barrier integrity. Caco-2 cells were grown on inserts and exposed to HS (40°C and 42°C) for 24h. The mRNA expression levels of HSP70 (A) and HSP90 (B) were measured by qRT-PCR after 6, 12 and 24h HS. TEER (C) and transport of Lucifer Yellow (LY) (D) from the apical to the basolateral chamber were measured. Data are presented as percentage of initial value (TEER), the amount of tracer transported [LY (ng/(cm^2 x h)] or relative mRNA expression as means ± SEM of three independent experiments, each accomplished in triplicate (*P < 0.05, **P < 0.01, ***P < 0.001; significantly different from the 37°C-exposed cells at the same exposure time).

GOS prevent the heat-induced upregulation of HSPs on mRNA and protein levels

Confluent Caco-2 cells were pre-incubated with GOS for 24h prior to 6h (qRT-PCR analysis) or 24h (western blot analysis and immunofluorescence staining) HS exposure. qRT-PCR analysis revealed that treatment with GOS significantly decreased HSP70 and HSP90 mRNA expression levels induced by 42°C heat treatment for 6h (Figs. 2A and 2B). Western blot analysis showed an increased protein expression of HSP70 and HSP90 after 24h HS exposure. The increased HSP70 protein levels were more pronounced after HS exposure as compared to HSP90 (Figs. 2C and 2D). Pre-treatment with GOS could concentration-dependently suppress the induction of HSP70 and HSP90 by thermal stress (Figs. 2C and 2D). The effect of GOS on the HS-induced HSP70 and HSP90 protein levels was confirmed by an immunofluorescence staining, since the HSP70 and HSP90 expression induced by HS was clearly decreased in the Caco-2 cells that were pre-incubated with GOS (Figs. 2E and 2F).

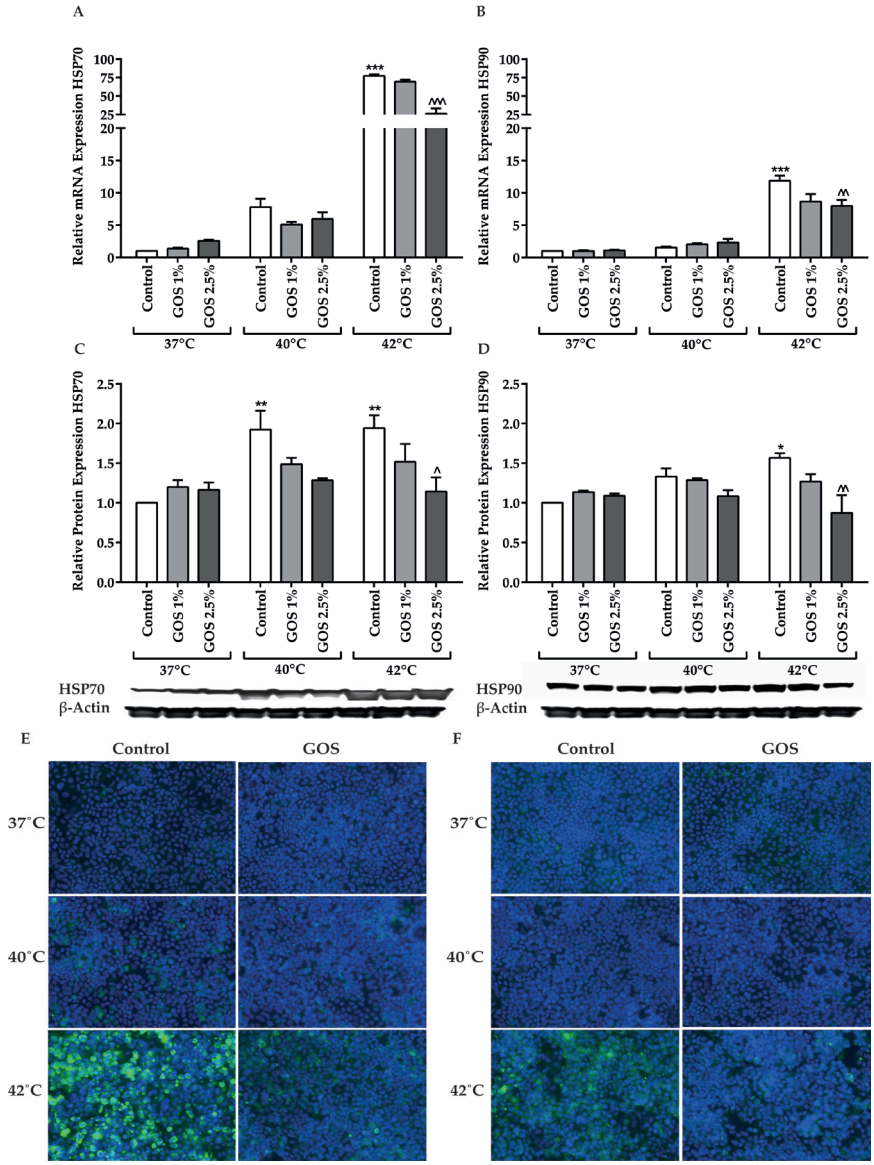

Figure 2. GOS prevent heat-induced upregulation of HSP70 and HSP90 on mRNA and protein level. Caco-2 cells grown on inserts were pre-treated with GOS (1% and 2.5%) and exposed to HS (40°C and 42°C) for 6h (qRT-PCR) or 24h (western blot analysis and immunofluorescence staining) to evaluate HSP70 and HSP90 mRNA (A,B), protein levels (C,D) or distribution pattern (E,F). Results are expressed as relative mRNA expression (qRT-PCR) or relative protein expression (western blot, optical density normalized with β-Actin) as mean ± SEM of three independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001; significantly different from the control cells exposed to 37°C. ^P < 0.05, ^^P < 0.01, ^^^P < 0.001; significantly different from the control cells exposed to 42°C). Results of the immunofluorescence staining with antibodies for HSP70 and HSP90 are presented at 200x magnification.

HS induces cellular oxidative stress and GOS suppress the heat-induced increase in mRNA expression of HO-1

HO-1 is considered as a cellular marker for oxidative stress, and to evaluate whether HS with or without GOS pre-treatment could affect the oxidative stress response, the mRNA expression of HO-1 was measured by qRT-PCR. Results indicated that 42°C HS for 6h resulted in an immediate upregulation of HO-1 as compared to the Caco-2 cells exposed to 37°C. Pre-treatment with GOS could dose-dependently suppress this induction of HO-1 mRNA expression levels (Fig. 3A).

A GOS-induced decrease in HSP levels does not lead to apoptosis

Since the heat shock protein expression can interfere with the process of apoptotic cell death, the effect of thermal stress on caspase-9 production was measured to exclude a direct effect of GOS on apoptosis. HS exposure (40°C and 42°C) to Caco-2 cells for 24h did not affect the caspase-9 protein expression measured by western blot analysis (Fig. 3B). However, a slight decrease in caspase-9 protein expression was observed in cells pre-treated with GOS, although these differences were statistically non-significant.

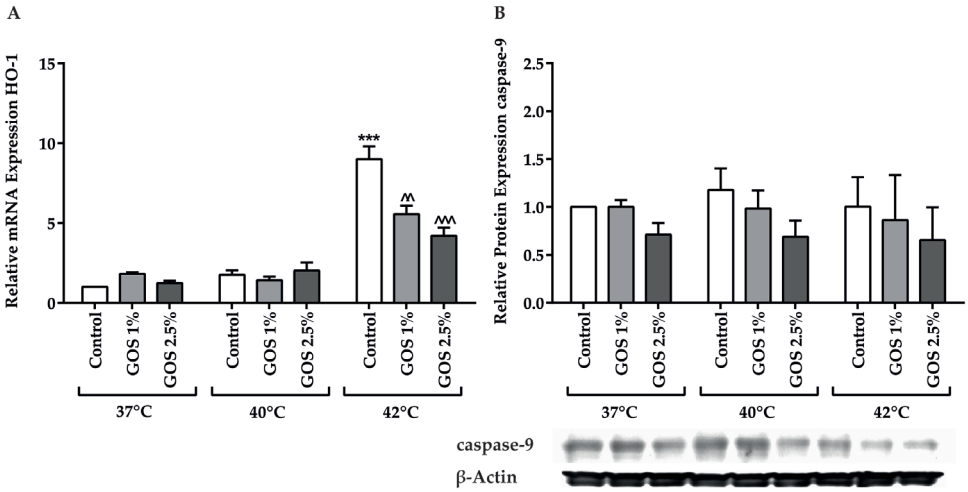

Figure 3. GOS suppress the heat-induced increase in HO-1 mRNA expression and do not affect caspase-9 protein expression. Caco-2 cells grown on inserts were pre-incubated with GOS for 24h and exposed to HS (40°C and 42°C) for 6h (qRT-PCR) to evaluate the mRNA expression of HO-1 (A) or for 24h (western blot) to measure the protein expression of caspase-9 (B). Results are expressed as relative mRNA expression (qRT-PCR) or relative protein expression (western blot, optical density normalized with β-Actin) as mean ± SEM of three independent experiments (***P < 0.001; significantly different from the control cells exposed to 37°C. ^^P < 0.01, ^^^P < 0.001; significantly different from control cells exposed to 42°C).

The heat-induced disruption of the intestinal barrier

Since thermal stress disrupts intestinal barrier integrity as observed in Figs. 1A and 1B, the effect of 24h GOS pre-incubation was investigated on the confluent Caco-2 cells exposed to HS for 24h. Results indicated that pre-treatment with GOS modulated the HS-induced TEER decrease in a dose-dependent manner (Fig. 4A). Besides the effect of GOS on 24h HS exposure, the TEER levels were also measured at earlier time points (2, 4, 6, 8, and 12h), where the effect of GOS was still present, albeit less pronounced (data not shown). Furthermore, pre-incubation with GOS could partly suppress the heat-induced increase in translocation of LY from apical to the basolateral side in 40°C and 42°C (Fig. 4B).

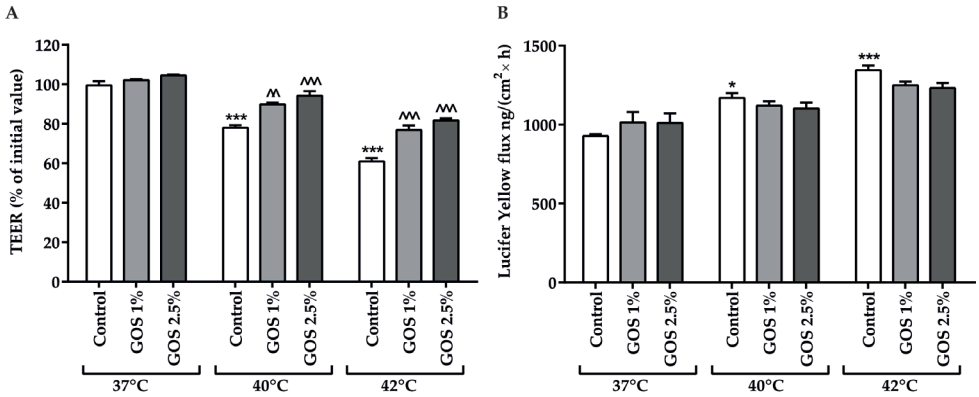

Figure 4. GOS partly prevent the heat-induced disruption of the intestinal epithelial barrier. Caco-2 monolayers grown on inserts were pre-treated with GOS and exposed to HS (40°C and 42°C) for 24h. TEER levels (A) as well as translocation of Lucifer Yellow (LY) (0.457 kDa) from the apical to the basolateral chamber (B) were measured. Data are presented as percentage of initial value (TEER) or in the amount of tracer transported [LY (ng/(cm^2 x h)] as means ± SEM of three independent experiments, each performed in triplicate (*P < 0.05, ***P < 0.001; significantly different from the control cells exposed to 37°C. ^^P < 0.01, ^^^P < 0.001; significantly different from corresponding control cells exposed to 40°C or 42°C).

HS modulates E-cadherin mRNA and protein level, while the tight junction (TJ) proteins remain unaffected

Since intestinal barrier integrity is intimately related with TJ and adherens junction (AJ) proteins, the mRNA expression levels of different TJ proteins (claudin-1, -3, -4, occludin and ZO-1) and E-cadherin, as typical AJ protein, were evaluated in Caco-2 cells exposed to HS. No significant changes in mRNA levels of the TJ proteins (claudin-1, -3, -4, occludin and ZO-1) were observed after thermal stress for 24h (Figs. 5A, 5B, 5C, 5D and 5E). However, after exposure to 42°C for 24h, a temperature-dependent upregulation in E-cadherin mRNA expression levels was observed (Fig. 5F). At the earlier time points of 6 and 12h heat exposure, no remarkable changes were detected in mRNA expression levels of TJs and E-cadherin (Fig. S2). The effect of HS exposure on TJ and AJ proteins in Caco-2 cells was further examined on

protein level via western blot analysis. Although thermal stress did not significantly affect the TJ protein expression, a slight decrease in occludin protein expression was observed after 24h HS (42°C) (Fig. S3). Interestingly, the E-cadherin protein levels were clearly decreased by exposure to 42°C (Fig. 6B).

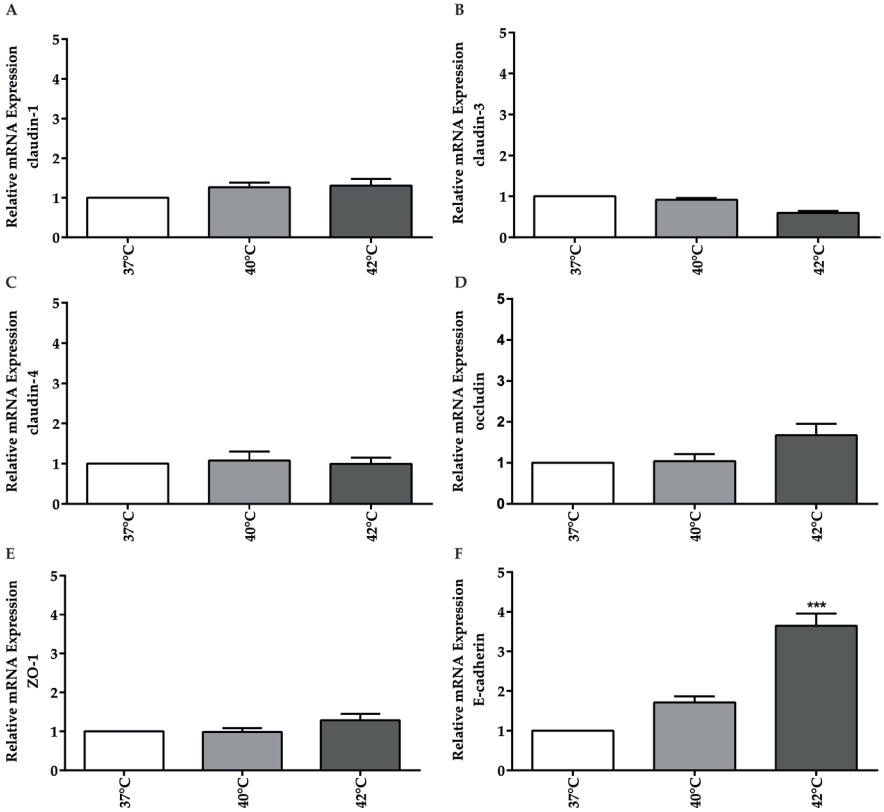

Figure 5. HS modulates E-cadherin mRNA expression, while the TJ proteins remain unaffected. Caco-2 cells were grown on inserts and exposed to different temperatures (37°C, 40°C and 42°C) for 24h. mRNA levels of TJ proteins claudin-1 (A), claudin-3 (B), claudin-4 (C), occludin (D), ZO-1 (E) and E-cadherin AJ (F) were measured by qRT-PCR. Results are expressing relative mRNA expression as mean ± SEM of three independent performed in triplicate (***P < 0.001; significantly different from the control cells exposed to 37°C).

GOS prevent the heat-induced effects on E-cadherin

HS did not significantly affect the TJ mRNA and protein expression, and hence pre-treatment with GOS resulted only in minor alterations, such as a tendency towards an enhancement of the protein expression of claudin-3 and occludin (Fig. S3). In contrast, qRT-PCR analysis indicated that pre-treatment with GOS significantly decreased the E-cadherin mRNA expression levels induced by 42°C heat treatment for 24h (Fig. 6A). While, a heat-induced decrease in E-cadherin protein expression was observed. The latter effects could be clearly prevented by pre-treatment with

GOS (Fig. 6B). The effect of GOS on the HS-induced decrease in E-cadherin protein levels was confirmed by an immunofluorescence staining, showing that E-cadherin was translocated from the cellular membrane to the submembraneous space. This irregular cellular distribution was partly prevented by GOS (Fig. 6C), as in the cells pre-treated with GOS and exposed to 42°C, more Caco-2 cell clusters were observed with E-cadherin localized at the cell membrane (red arrows) compared to the heat-exposed cells without pre-treatment.

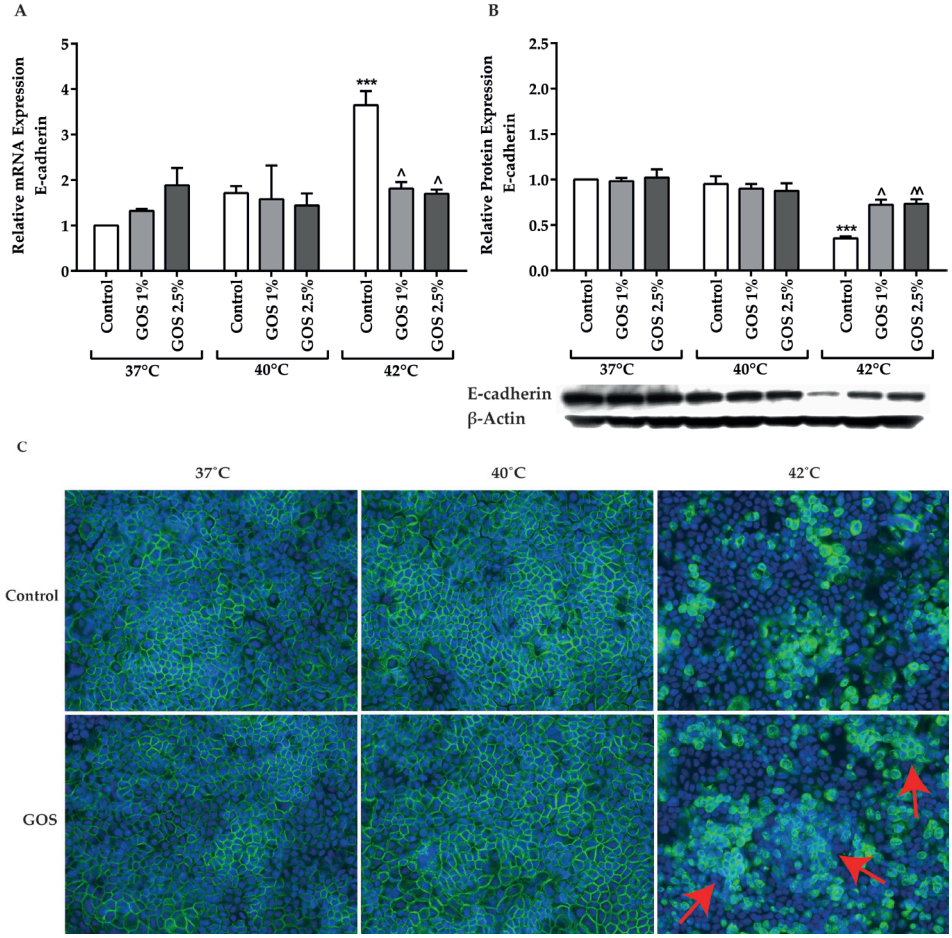

Figure 6. GOS prevent the heat-induced effects on the E-cadherin. Caco-2 cells grown on inserts were pre-incubated with GOS (1% and 2.5%) and exposed to HS (40°C and 42°C) for 24h. The expression of E-cadherin in mRNA level (A), protein level (B) and cellular distribution (C) was evaluated. Results are expressed as relative mRNA expression (qRT-PCR) or relative protein expression (western blot, optical density normalized with β-Actin) as mean ± SEM of three independent experiments (***$P < 0.001$; significantly different from the control cells exposed to 37°C. ^$P < 0.05$, ^^$P < 0.01$; significantly different from the control cells exposed to 42°C). Immunofluorescence results are performed using an antibody for anti E-cadherin and are presented at 200x magnification.

Discussion

Non-digestible oligosaccharides, like GOS, are known as functional food ingredients, that can modify the gut function by enhancing the growth of beneficial bacteria, stimulating immune responses and maintaining the intestinal barrier integrity [12,15,16]. Therefore, we hypothesized that dietary GOS could protect the intestinal epithelial barrier against heat-induced effects on intestinal barrier integrity, oxidative stress and associated HSP production.

HSPs have been recognized as markers of thermal stress [9,17]. In our *in vitro* model, thermal stress of 42°C induced an increase in HSP70 and HSP90 mRNA and protein expression in Caco-2 cells, which was prevented by pre-incubation of the cells with GOS. These effects were confirmed by immunofluorescence staining for HSP70 and HSP90. The HSP70 mRNA and protein expression in Caco-2 cells was more pronounced after thermal stress as compared to HSP90. HSP70 is known as the most temperature sensitive and highly conserved member of the HSP family [18]. Previous *in vitro* studies in K562 cells also indicated that exposure to high temperatures would induce significantly more HSP70 and to a lesser extent HSP90 [19,20]. HSP70 controls in a non-specific manner protein folding, while HSP90 interacts with specific client proteins such as hormone receptors and protein kinases {reviewed in [21,22]}. These differences could be possible explanations for differences in HSP70 and HSP90 expression after thermal stress. Our findings related to the HSP expression are in line with Dokladny *et al.* who also showed a significant increase in protein expression of HSP70 and HSP90 in Caco-2 cells exposed to 41°C for 24h [9]. Various previous studies focused on strategies to decrease HSPs to stimulate apoptosis, or increase HSP to suppress cell death, depending on the pathological conditions, as for example in cancer therapy, induction of apoptosis might be beneficial [23].

In the intestines and many other organs, HSP70 and HSP90 are known as negative regulators of apoptosis during stressful conditions and interact with the caspase-mediated death signalling pathways by inhibiting effector-caspases [5,24]. To exclude that decreasing HSP70 and HSP90 expression levels induced by GOS in our study were not provoking apoptosis of the Caco-2 cells, the expression of caspase-9 was measured. Our results demonstrated that the decreased expression of HSPs caused by GOS pre-treatment did not induce apoptosis through an upregulation of caspase-9 protein levels. It has previously been shown that within the caspase-mediated apoptosis chain, caspase-9 activation is strictly required for apoptosis induced by thermal stress in Jurkat cells [23].

HSPs are also considered as sensitive biomarkers that directly represent the degree of oxidative stress [25]. Previous studies demonstrated already the protective effect of radical scavengers against oxidative stress resulted in suppressed expression of HSPs in different cell lines [26,27]. Another, independent and sensitive marker of oxidative stress is HO-1. Comparable to the HSPs, HO-1 is known to

be transcriptionally induced in response to heat exposure in intestinal cells [28]. Hence we measured the relative mRNA expression of HO-1 and indeed could show that exposure to 42°C significantly induced HO-1 expression in Caco-2 cells and GOS prevented the heat-induced increase in HO-1 mRNA levels. A protective role of GOS against oxidative stress was also reported in T84 intestinal cells [29]. Moreover, *in vivo* supplementation of diets with fructo-oligosaccharides (FOS) or GOS alleviated the oxidative stress injury in hepatocytes and renal cells [30–32]. Although in these *in vivo* studies, the protecting role of oligosaccharides against oxidative stress has been dedicated to the improvement of the intestinal microflora, we here demonstrated that also a direct cell-protective effect should be considered.
It is already known that exposure to thermal stress can induce gut barrier dysfunction and increase intestinal epithelial permeability to luminal antigens and lead to bacterial translocation through the gut epithelium [33–35]. In line with these findings, our results showed a temperature- and time-dependent decrease in TEER levels and temperature-dependent increase in paracellular permeability to LY, indicating physical impairment of the cell monolayer. The integrity of the intestinal barrier largely depends on the expression and function of TJ and AJ proteins facilitating the efficient cell-cell adhesion [36]. Previous studies indicated that the heat-induced increase in paracellular permeability is mainly associated with TJ proteins assembly [9,33]. Xiao *et al.* described that the occludin and ZO-1 mRNA and protein levels were decreased in Caco-2 cells exposed to severe thermal-stress for 1h (43°C) and N-3 polyunsaturated fatty acids protected this heat-induced permeability dysfunction [10]. In contrary to these previous reports, we did not find any significant effects on TJ protein mRNA or protein expression after HS exposure. These differences suggest that different heat exposure times, temperatures and recovery periods may result in different expression levels of TJ proteins. Nevertheless, GOS pre-treatment could partly prevent the thermal stress-induced decrease in TEER levels as well as LY translocation from the apical to the basolateral compartment of the Caco-2 monolayer. qRT-PCR analysis of E-cadherin showed an upregulation in mRNA levels after HS, which may be recognized as a compensatory response to HS-induced changes in E-cadherin protein levels. Further experiments demonstrated that HS could markedly reduce the protein expression and altered the cellular localization of E-cadherin. These results confirmed previous findings indicating that heat shock could alter cell-cell adhesion by downregulating E-cadherin protein levels [37]. Recently, Chen *et al.* demonstrated that the underlying mechanism of E-cadherin expression during thermal stress corresponds with the HSP90-induced TCF-12 gene expression as negative regulator of E-cadherin protein [38]. Moreover, our results clearly indicated the HS-induced mRNA expression, protein derangement and delocalization of E-cadherin could be prevented by GOS pre-treatment.
Further research is needed to unravel the mechanisms behind this microbiota-independent protective effect of GOS. *In vivo* HS models with chickens also reported

that mannan-oligosaccharides and probiotic mixtures can partially lessen the heat-induced changes in intestinal morphology and intestinal barrier function [39]. Moreover, Xu *et al*. showed that a certain size of oligosaccharides, tetrasaccharides of hyaluronan, upregulated HSP72 expression under HS conditions and suppressed cell death [40]. This finding suggests that the size of oligosaccharides could be important for the HSP-related effect. The currently applied GOS, is a complex mixture of oligosaccharides produced with DP and glycosidic linkages [41]. It remains to be elucidated if there are significant differences in the biological activity of GOS related to the structures, molecular weight and type of glycosidic linkages. As yet it is only known that non-digestable oligosaccharides, like GOS, might interact with peptidoglycan recognition protein 3, peroxisome proliferator-activated receptor γ or carbohydrate receptors, such as C-type lectin [13,42,43]. Furthermore, there might be a similarity between the protection of GOS against HS and the thermal protection of fungal species by accumulation of high levels of trehalose-based oligosaccharides [44]. Trehalose may act as an antioxidant and is capable of reducing protein carbonylation during oxidative stress. It also stabilizes the structure of lipid bilayers and of proteins, avoids protein aggregation, and prevents (oxidative) changes in large molecules {reviewed by [45]}. Possibly, GOS, like other sugars, have a macromolecule-stabilizing character that protect cells against oxidative and HS [46].

Conclusions

Our results indicate that galacto-oligosaccharides protect the intestinal epithelial barrier against HS as observed by a decrease in heat-induced HSP70 and HSP90 on mRNA and protein levels, and by a suppression of the heat-induced oxidative stress response. Furthermore, in the absence of measureable changes in expression of TJ proteins, thermal stress-induced disruption of the intestinal epithelial barrier can be particularly associated with the derangement of E-cadherin, which is mitigated by pre-treatment of cells with GOS. It can be concluded that dietary GOS, nowadays mainly used in infant formulas, may be an attractive dietary application for people who are at high risk of developing HS. However, further investigations are necessary to identify the molecular targets of oligosaccharides (from different origins) in the intestines and to establish a structure-activity relationship.

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Supporting Information

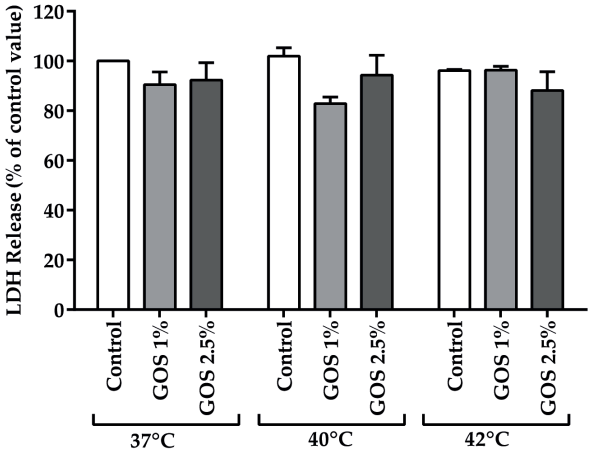

Figure S1. HS does not affect cell viability. Caco-2 monolayers grown on inserts were pre-treated with GOS (1% and 2.5%) before being exposed to different temperatures (37°C, 40°C and 42°C) for 24h, followed by measurement of LDH release in the apical compartment of the transwell insert system. Results are expressed as percentage of LDH released by the control cells exposed to 37°C as mean ± SEM of three independent experiments, each performed in triplicate.

Table S1. Primer sequences used for qRT-PCR

	Primer sequence (5′-3′)			
Genes	**Forward**	**Reverse**	**AT**	**References**
β-Actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	63	NM_001101
claudin-1	AGCTGGCTGAGACACTGAAGA	GAGAGGAAGGCACTGAACCA	63	NM_021101
claudin-3	CTGCTCTGCTGCTCGTGTC	CGTAGTCCTTGCGGTCGTAG	63	NM_001306
claudin-4	GTCTGCCTGCATCTCCTCTGT	CCTCTAAACCCGTCCATCCA	62.5	NM_001305
E-cadherin	TGGACCGAGAGAGTTTCCCT	CCCTTGTACGTGGTGGGATT	60	BC-144283.1
HO-1	GCCACCAAGTTCAAGCAGCT	CAGTGCCCACGGTAAGGAAG	61.2	NM_002133.2
HSP70	AGAGCCGAGCCGACAGAG	CACCTTGCCGTGTTGGAA	57	NG_011855.1
HSP90	CCTTCTATTTGTCCCACG	ATCCTCCGAGTCTACCAC	58.7	NM_005348.3
occludin	TTGGATAAAGAATTGGATGACT	ACTGCTTGCAATGATTCTTCT	57	NM_002538
ZO-1	GAATGATGGTTGGTATGGTGCG	TCAGAAGTGTGTCTACTGTCCG	55.8	NT_010194.17

AT, Annealing temperature (°C)

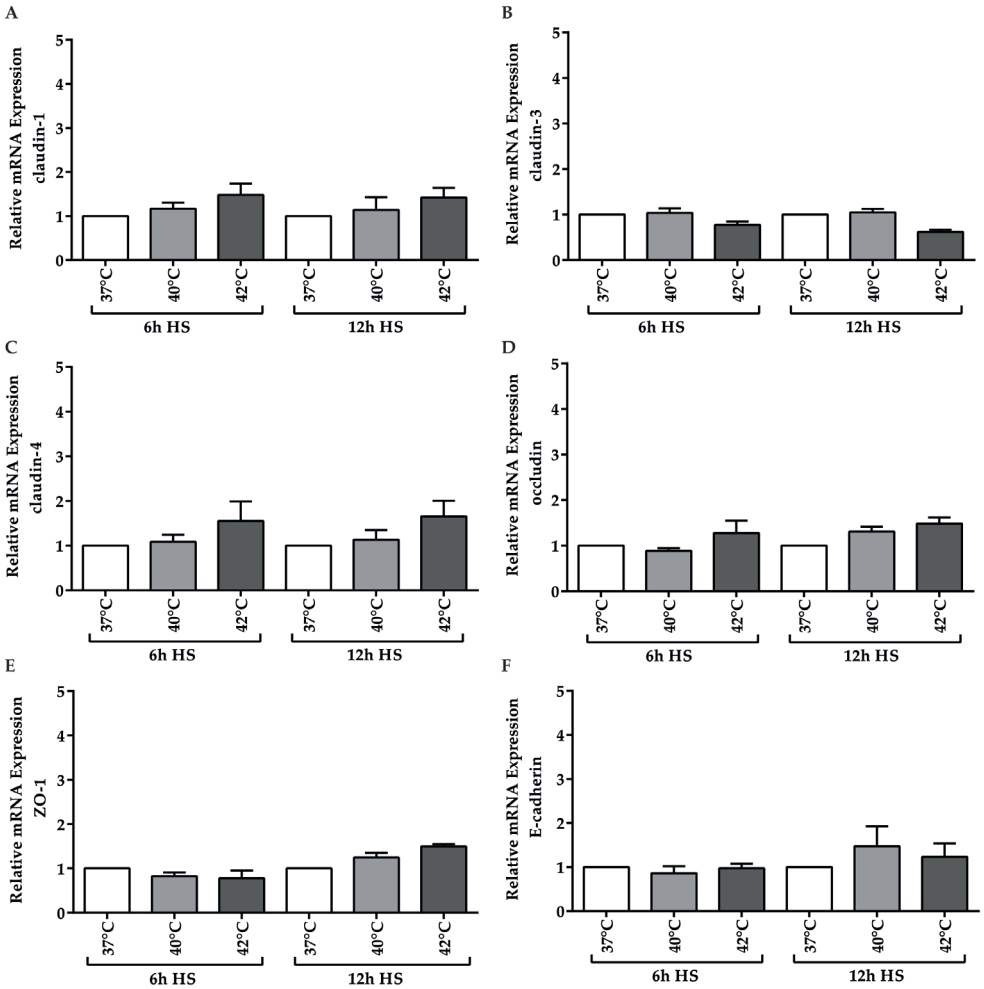

Figure S2. Thermal stress selectively but not significantly modulates the TJ proteins and E-cadherin at early time points. Caco-2 cells were grown on inserts and after pre-treatment with GOS (1% and 2.5%) for 24h, exposed to HS (40°C and 42°C) for 6 and 12h. mRNA levels of TJ proteins claudin-1 (A), claudin-3 (B), claudin-4 (C), occludin (D), ZO-1 (E) and E-cadherin (F) were measured by qRT-PCR. Results are expressed as relative mRNA expression as mean ± SEM of three independent experiments each performed in triplicate.

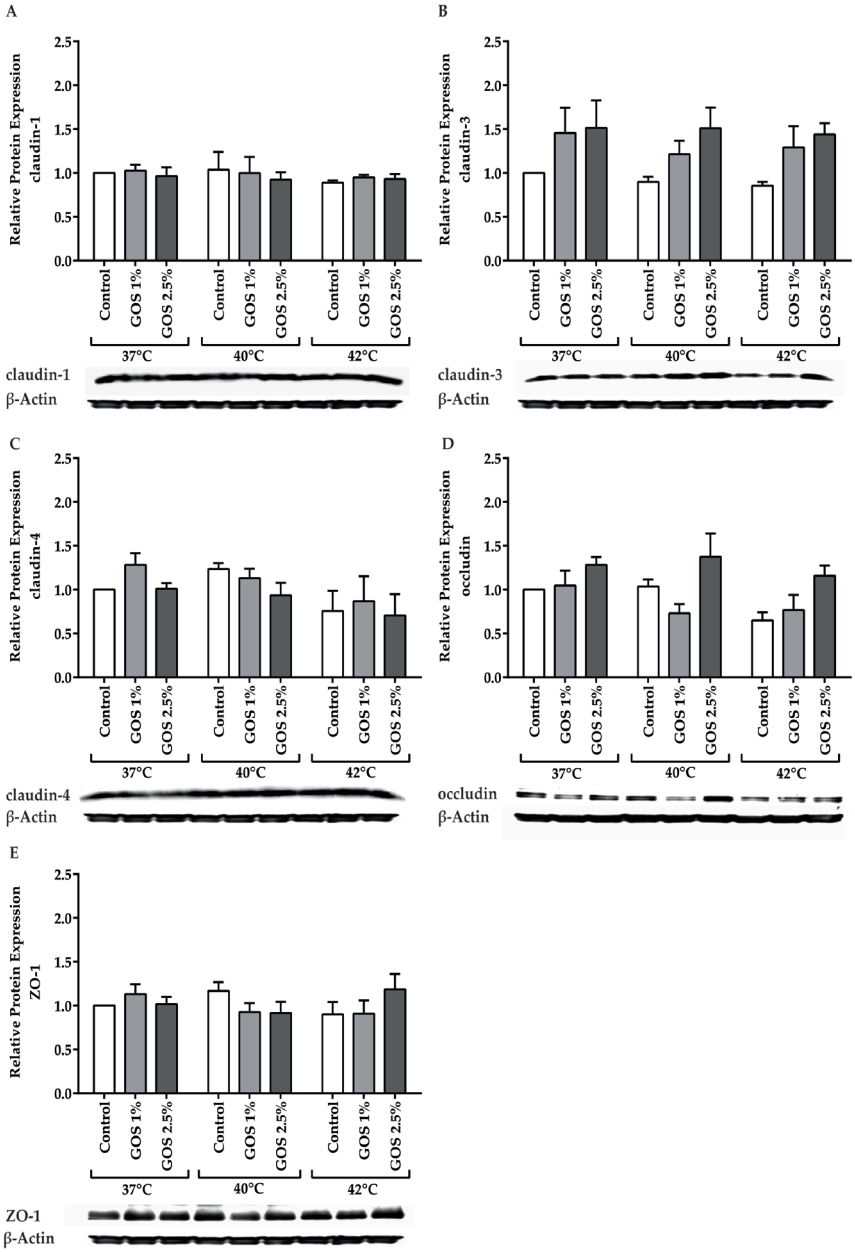

Figure S3. GOS can partly modulate the expression of TJ proteins claudin-3 and occludin during thermal stress. Caco-2 cells were grown on inserts and after pre-treatment with GOS (1% and 2.5%) for 24h, exposed to HS (40°C and 42°C) for 24h. The protein expression of TJ proteins claudin-1 (A), claudin-3 (B), claudin-4 (C), occludin (D), ZO-1 (E) were measured by western blot analysis. Results are expressed as relative protein expression (western blot, optical density normalized with β-Actin) of three independent experiments.

Chapter 4

Differences in susceptibility to heat stress along the chicken intestine and the protective effects of galacto-oligosaccharides

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Abstract

High ambient temperatures negatively affect the human well-being as well as animal welfare and production. The gastrointestinal tract is predominantly responsive to heat stress (HS). The currently available information about the multifaceted response to HS within different parts of the intestine is limited, especially in avian species. Hence, this study aims to evaluate the HS-induced sequence of events in the intestines of chickens. Furthermore, the gut health-promoting effect of dietary galacto-oligosaccharides (GOS) was investigated in these HS-exposed chickens.
Chickens were fed a control diet or diet supplemented with 1% or 2.5% GOS (6 days) prior to and during a temperature challenge for 5 days (38-39°C, 8h per day). The parameters measured in different parts of the intestines included the genes (qRT-PCR) HSF1, HSF3, HSP70, HSP90, E-cadherin, claudin-1, claudin-5, ZO-1, occludin, Toll-like receptor (TLR)-2, TLR-4, IL-6, IL-8, haem oxygenase-1 (HO-1), hypoxia inducible factor, subunit alpha (HIF-1α) and their associated proteins HSP70, HSP90 and pan-cadherin (western blots). In addition, IL-6 and IL-8 plasma concentrations were measured by ELISA. In the jejunum, HSF3, HSP70, HSP90, E-cadherin, claudin-5, ZO-1, TLR-4, IL-6 and IL-8 mRNA expression and HSP70 protein expression were increased after HS exposure and a more pronounced increase in gene expression was observed in ileum after HS exposure, and in addition HSF1, claudin-1 and HIF-1α mRNA levels were upregulated. Furthermore, the IL-8 plasma levels were decreased in chickens exposed to HS. Interestingly, the HS-related effects in the jejunum were prevented in chickens fed a GOS diet, while dietary GOS did not alter these effects in ileum. In conclusion, our results demonstrate the differences in susceptibility to HS along the intestine, where the most obvious modification in gene expression is observed in ileum, while dietary GOS only prevent the HS-related changes in jejunum.

Introduction

Heat stress (HS) is one of the most relevant environmental stressors in poultry production worldwide [1]. It has been suggested that in modern poultry genotypes the rapid growth rate is responsible for the reduction in heat tolerance due to the higher metabolic activity [2–4]. In turn, today's chickens seem to be particularly susceptible to high environmental temperatures and suffer from multiple pathophysiological alterations, such as immune dysregulation, gut barrier dysfunction and cellular oxidative stress after heat exposure, resulting in decreased productivity and increased susceptibility to infectious diseases and higher mortality [5–7]. Response to environmental stressors, including HS, starts with the phosphorylation and trimerisation of heat shock factors (HSF) and these trimers translocate to the nucleus and bind the so-called heat shock elements in the promoter region of heat shock protein (HSP) genes, mediating HSP gene transcription. HSPs play a pivotal role in repair and protection of the internal environment by assisting protein refolding and by promoting the degradation of misfolded proteins [8,9]. A general symptom of HS is the disturbance of the balance between the production of reactive oxygen species and the cellular antioxidant defences, resulting in oxidative stress [4,10]. The gastrointestinal tract is primarily responsive to HS and a variety of changes can be observed, including alterations in the microbiota and an impairment of intestinal barrier integrity [10,11]. These changes allow the translocation of luminal antigens and pathogens through the intestinal epithelium and facilitate the response of the innate immune system by exaggerating the extent of Toll-like receptor (TLR) signalling, ultimately leading to the development of intestinal inflammation and damage [12,13]. In addition, HSPs are recognized by TLRs in many cell types and can directly initiate an inflammatory response [14–16]. Moreover, the intestinal barrier integrity can be affected by different cytokines [17] and an increase in pro-inflammatory cytokines, like IL-6 and IL-8, has been observed in intestinal epithelial cells after barrier disruption [18,19]. It is also known that the upregulation of HSPs, and in particular HSP70, is considered to be a protective mechanism as they can also inhibit the expression of pro-inflammatory cytokines [20,21]. The HS-induced damages within the intestine is a complex process and needs to be investigated in order to identify intervention strategies and hence, this study focused on the assessment of typical alterations in the expression of a number of genes and their corresponding proteins, such as HSFs, HSPs, adherens junctions (AJ) and tight junctions (TJ), TLRs, cytokines/chemokines and oxidative stress markers, which are all related to the hypothetical cascade of events occurring in different parts of the intestine from broilers upon HS exposure. Previous intervention strategies to alleviate HS in poultry mainly focused on improvement in antioxidant capacity attributed to supplementation with selenium, vitamins and different unsaturated acids, including α-lipoic acid [22–25]. In contrast, limited information is available about promoting gut health

and intestinal barrier integrity in HS susceptible chickens. Food supplementation with prebiotics, including galacto-oligosaccharides (GOS) are known to support the maintenance of the gut homeostasis, not only by increasing the beneficial bacteria population, but also by directly improving gut barrier functions and gut-associated immunity [19,26–28]. Therefore, this study included also experiments aiming to evaluate the possible protective role of GOS against the HS-induced alterations in the intestines of chickens.

Materials and Methods

Animals and experimental design

Sixty 15-day-old Ross broilers were randomly divided into 6 groups (control group, control group + 1% GOS, control group + 2.5% GOS, HS group, HS group + 1% GOS, HS group + 2.5% GOS). The chickens were housed in two environmentally controlled chicken rooms (control and HS room) equipped with 3 special bird units and equipped with a lighting program of 16h light and 8h dark per day. After an acclimatization period of 6 days (22-26°C), the control groups maintained the ambient temperature of 22-23°C, while the room temperature for the HS group was 38-39°C for 8h during the daylight period for 5 consecutive days, and 22-23°C during the remaining period. All chickens were provided with free access to water and feed. The whole experimental period (day 1-11), the broilers were fed either a standard broiler diet (Table S1) or the standard diet supplemented with 1% or 2.5% GOS (Research Diet Services, Wijk-bij-Duurstede, The Netherlands). GOS was obtained from FrieslandCampina Domo (Vivinal® GOS syrup, Borculo, The Netherlands) containing oligosaccharides with a degree of polymerisation (dp) of 2-8 with approximately 59% (w/w) galacto-oligosaccharides, 21% (w/w) lactose, 19% (w/w) glucose and 1% (w/w) galactose on dry matter (dry matter of 75%).
The experimental protocol was established in line with the prerequisites of the use of animals in research (DIRECTIVE 2010/63/EU) and had been approved prior to the onset of the experimental trials by the animal welfare committee of the University of Veterinary Medicine Hannover (competent ethics committee of the university) on the uses of animals in research according to BGBl. I S. 1105/2.

Sample collection

At the end of the experimental period, the animals were sacrificed by cervical dislocation and immediately after decapitation blood was collected. Samples from the duodenum (3 cm after gizzard), jejunum (5 cm before Meckel's diverticulum), ileum (5 cm before ileo-cecal transition), cecum (proximal part) and colon (5 cm after ileo-cecal transition) were dissected, directly rinsed in phosphate buffer saline (PBS), snap frozen in liquid nitrogen and stored at -80°C for qRT-PCR or western blot analysis. Plasma was derived from blood (~10 ml), harvested by centrifugation (15 minute at 1500 × g) and stored at –20°C.

Quantitative RT-PCR (qRT-PCR) analysis

Intestinal specimens suspended in RNA lysis buffer containing β-mercaptoethanol were homogenized using the TissueLyser (Qiagen, Hilden, Germany) for 1 minute/25 Hz and total RNA was isolated using spin columns based on manufacturer's instructions (Promega, Madison, WI, USA). RNA was reverse-transcribed to cDNA using iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The PCR reaction mixture, containing iQSYBR Green Supermix (Bio-Rad) was prepared based on

manufacturer's instructions and qRT-PCR analysis was performed using the MyiQ single-colour real time PCR detection system (Bio-Rad) with MyiQ System Software Version 1.0.410 (Bio-Rad). Commercially manufactured gene specific primers (Eurogentec, Seraing, Belgium) were used after confirmation of specificity and efficiency tests by qRT-PCR with dilution series of pooled cDNA at a temperature gradient (55°C to 65°C) for primer-annealing and subsequent melting curve analysis (Table S2). The mRNA quantity was calculated relative to the expression of β-Actin reference gene.

Western blot analysis

Approximately, 50 mg of jejunum and ileum specimens (five randomly selected samples per group) were lysed with 500 μl RIPA lysis buffer (Thermo scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany). Total protein concentration was assessed by a BCA protein assay kit (Thermo scientific) and equal protein amounts of boiled samples were separated by electrophoresis (Criterion™ Gel, 4-20% Tris-HCL, Bio-Rad) and electro-transferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with PBS supplemented with 0.05% Tween-20 (PBST) and 5% milk proteins and incubated overnight at 4°C with antibodies for HSP70 (1:2000 Abcam, Cambridge, UK), HSP90 (1:1000, Enzo Life Sciences, Farmingdale, NY, USA), and pan-cadherin (1:1000, Abcam). Membranes were subsequently probed with an anti-β-Actin antibody (1:2000, Cell Signaling, Danvers, MA, USA) to evaluate equality of loading. After washing in PBST, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000, Dako, Glostrup, Denmark) for 2h at room temperature. Finally, blots were washed in PBST, incubated with ECL Prime western blotting detection reagent (Amersham Biosciences, Roosendaal, The Netherlands) and digital images were obtained with the ChemiDoc™ MP imager (Bio-Rad). Signal intensities were quantified using the ImageJ 1.47 software and the protein expression was normalized with β-Actin and expressed as mean fold change in relation to the control group.

Cytokine measurement

IL-8 and IL-6 concentrations were measured in plasma and jejunal and ileal homogenates using the CXCL8-ELISA kit (BD Biosciences, Cat.# 555244, San Diego, CA, USA) and IL-6 ELISA kit (MyBiosources, Cat.# MBS037319, San Diego, CA, USA) according to manufacturer's instructions.

Statistical analysis

Experimental results are expressed as mean ± SEM of n=6-10 animals/experiment (qRT-PCR and ELISA) or n=5 animals/experiment (western blot analysis). Differences between multiple groups (qRT-PCR and ELISA results) were statistically determined by using Two-way ANOVA, with Bonferroni posthoc test,

while data of the western blot analysis were statistically analysed by using One-way ANOVA followed by a Bonferroni post-hoc test. Analyses were performed by using GraphPad Prism (version 6.05) (GraphPad, La Jolla, CA, USA) and results were considered statistically significant when $P < 0.05$. Different lowercase letters on the bars indicate significant differences between groups.

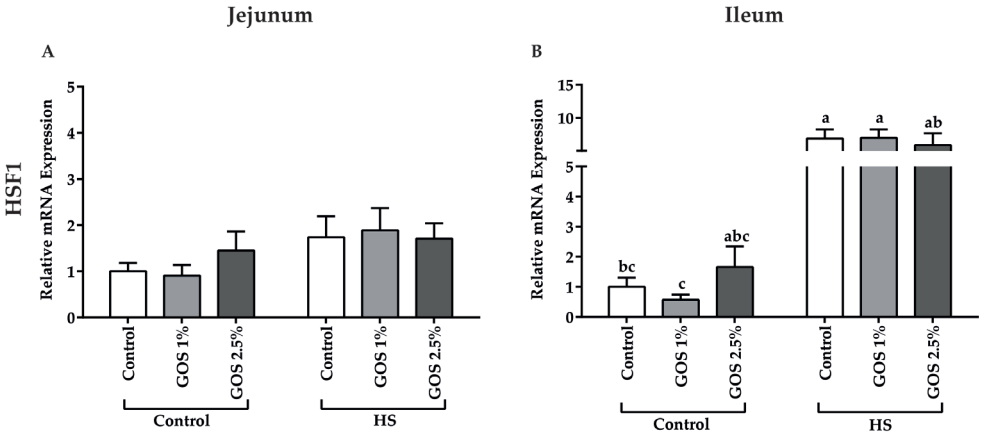
Results

The effect of HS on HSF1, HSF3, HSP70 and HSP90 mRNA levels is more pronounced in ileum compared to jejunum, while dietary GOS only prevent the HS response (HSR) in jejunum

HS resulted in a significant mRNA upregulation of HSF3 in the chicken jejunum (Fig. 1C), while in ileum both HSF1 and HSF3 mRNA levels were increased after heat exposure (Figs. 1B and 1D). The mRNA expression of HSP70 and HSP90 was significantly upregulated in both jejunum and ileum of chickens exposed to HS compared to control chickens (Figs. 1E, F, G, H), although the effect on HSP70 was more pronounced (Figs. 1E and 1F). In general, the induction of the HSR observed by expression of HSFs and HSPs, was more obvious in the chicken ileum in comparison with the jejunum (Fig. 1). In addition, no significant effects on HSP70 and HSP90 mRNA levels were observed in duodenum and colon of chickens exposed to HS (Fig. S1). Dietary GOS prevented the HS-induced upregulation of HSF3 (Fig. 1C), HSP70 (Fig. 1E) and HSP90 (Fig. 1G) in the chicken jejunum, while no preventive effect of GOS was observed on the HSR in the ileum of chickens exposed to HS (Figs. 1B, D, F, H).

HSP70 protein levels are increased in jejunum and ileum after HS exposure and dietary GOS prevent this effect in jejunum

Results indicated that the protein expression of HSP70 was significantly increased in jejunum and ileum of chickens exposed to HS (Figs. 2A and 2B), whereas the HSP90 protein levels did not change in response to HS (Figs. 2C and 2D). Dietary GOS could prevent the HS-induced increase in HSP70 protein expression in chicken jejunum (Fig. 2A), while in the ileum a small but not significant decrease was observed in the HS-induced HSP70 protein levels (Fig. 2B).



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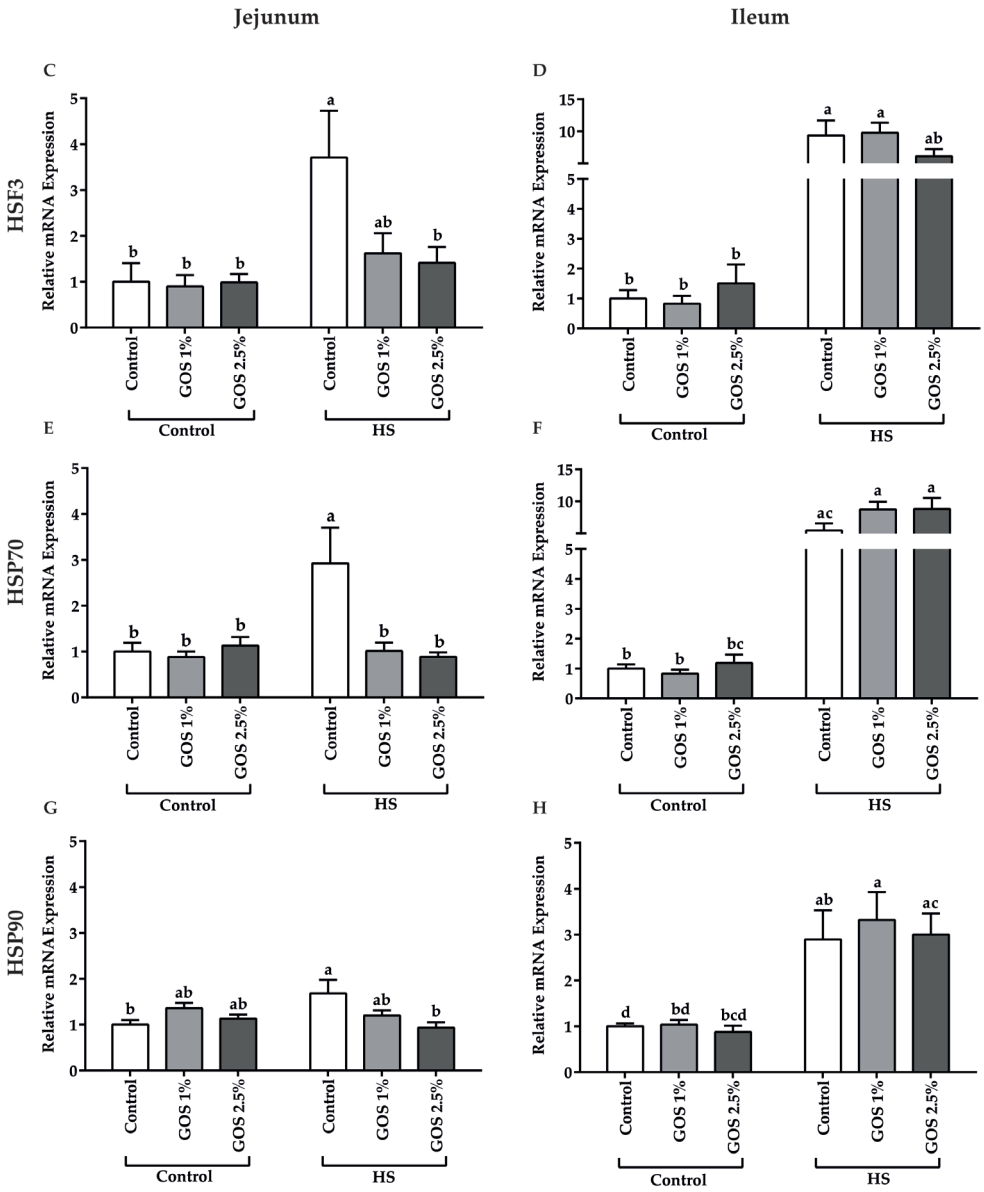


Figure 1. The effect of HS exposure on the mRNA expression of HSFs and HSPs in jejunum and ileum of chickens fed a control or GOS diet. Chickens fed a control or GOS (1 or 2.5%) diet for 6 days before being exposed to either control or HS conditions for 5 days. mRNA expression levels of HSF1 (A,B), HSF3 (C,D), HSP70 (E,F) and HSP90 (G,H) were evaluated in jejunum (A,C,E,G) and in ileum (B,D,F,H) by qRT-PCR. Results are expressed as relative mRNA expression (fold of control, normalized to β-Actin) as mean ± SEM, n=6-10 animals/ experimental group. Different lower-case letters denote significant differences among groups.

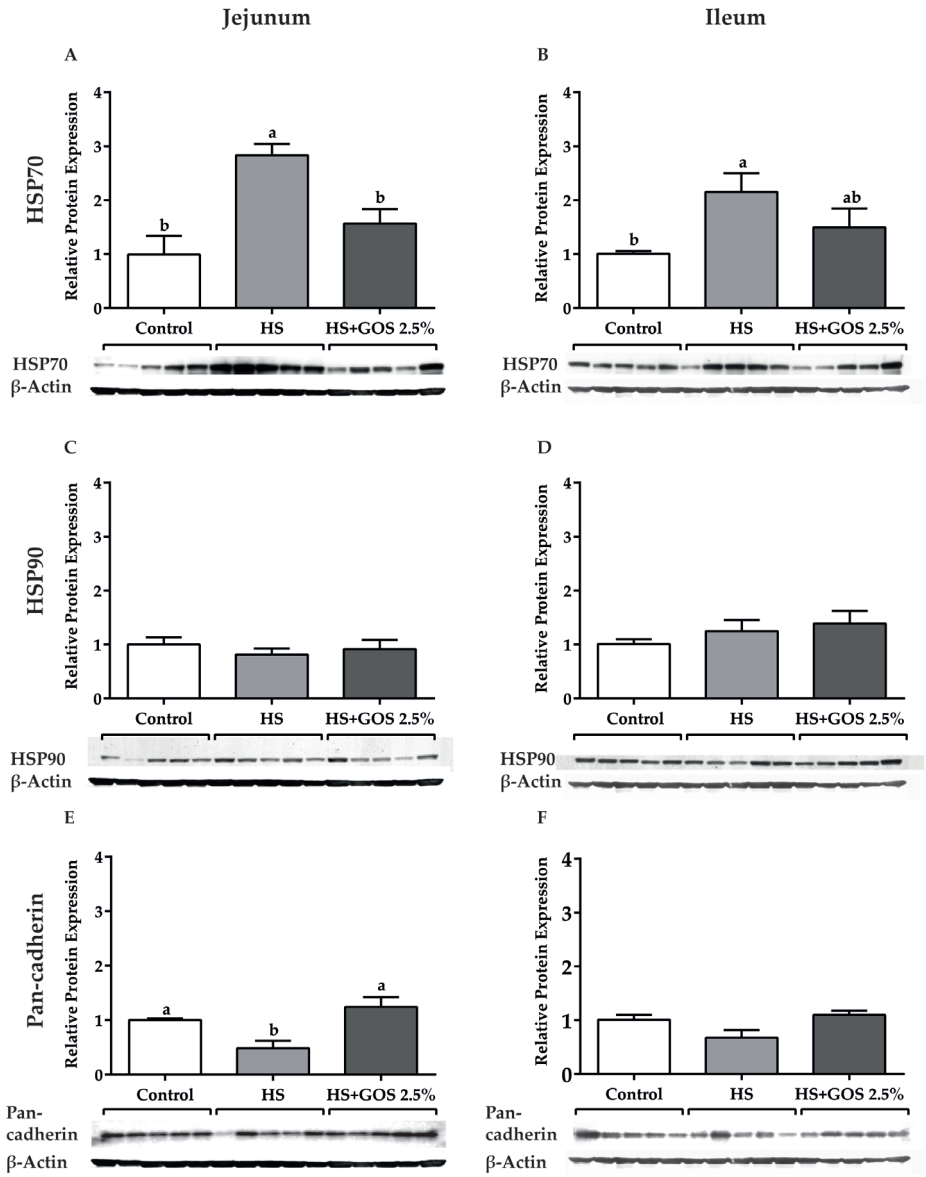


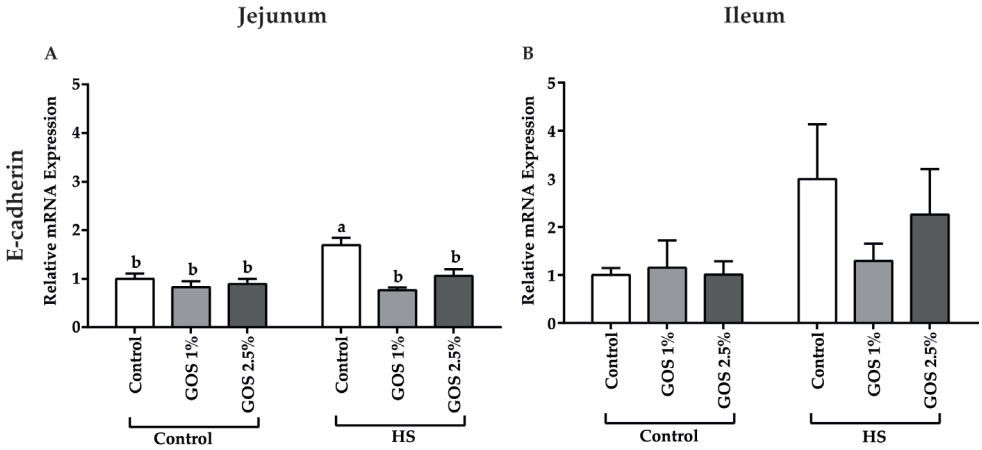
Figure 2. The effect of HS exposure on the protein expression of HSPs and pan-cadherin in jejunum and ileum of chickens fed a control or GOS diet. Chickens fed a control or GOS 2.5% diet for 6 days before being exposed to control or HS conditions for 5 days. Protein expression levels of HSP70 (A,B), HSP90 (C,D) and pan-cadherin (E,F) were measured in jejunum (A,C,E) and ileum (B,D,F) by western blot analysis (lane 1-5: control chickens, lane 6-10: heat-exposed chickens, lane 11-15: heat-exposed chickens fed a GOS diet). Results are expressed as relative protein expression (fold of control, normalized to β-Actin) as mean ± SEM. n=5 animals/experimental group. Different lower-case letters denote significant differences among groups.

HS modulates the E-cadherin and TJ protein mRNA expression in the small intestine and dietary GOS prevent this effect in jejunum

HS significantly upregulated the E-cadherin mRNA expression in chicken jejunum (Fig. 3A), while in ileum the heat-induced increase in E-cadherin mRNA levels was not statistically significant (P=0.94) (Fig. 3B). The western blot analysis revealed that HS decreased the pan-cadherin protein expression in jejunum (Fig. 2E), although again no significant effect in the ileum was observed (Fig. 2F). The heat-induced effects on both the E-cadherin mRNA expression and pan-cadherin protein expression in jejunum were prevented in chickens fed a GOS diet compared to chickens fed a control diet (Figs. 2E and 3A). Besides E-cadherin, the mRNA expression levels of the TJ proteins claudin-5 (Fig. 3E) and zona occludens protein-1 (ZO-1) (Fig. 3G) were significantly increased in jejunum of chickens exposed to HS. A more pronounced increase in claudin-5 (Fig. 3F) and ZO-1 (Fig. 3H), but also claudin-1 (Fig. 3D) was observed in the ileum after HS exposure. HS did not induce any changes in claudin-1 mRNA expression in jejunum (Fig. 3C) and occludin mRNA expression in both jejunum and ileum (Fig. S2). In contrast to the ileum, the heat-induced increase in TJ mRNA expression (claudin-5 and ZO-1) was dose-dependently alleviated in the jejunum of chickens fed a GOS diet (Figs. 3E and 3G).

TLR-4 mRNA levels are increased in jejunum and ileum after HS exposure and dietary GOS counteract this effect in jejunum

Although no significant differences were detected in TLR-2 mRNA levels between control and heat-exposed chickens in jejunum and ileum (Figs. 4A and 4B), the mRNA expression of TLR-4 increased significantly in both jejunum (Fig. 4C) and ileum (Fig. 4D) after heat exposure. Supplementation of GOS (2.5%) to the diet significantly prevented the heat-induced TLR-4 induction in jejunum (Fig. 4C), while GOS did not modulate this effect in the chicken ileum (Fig. 4D).



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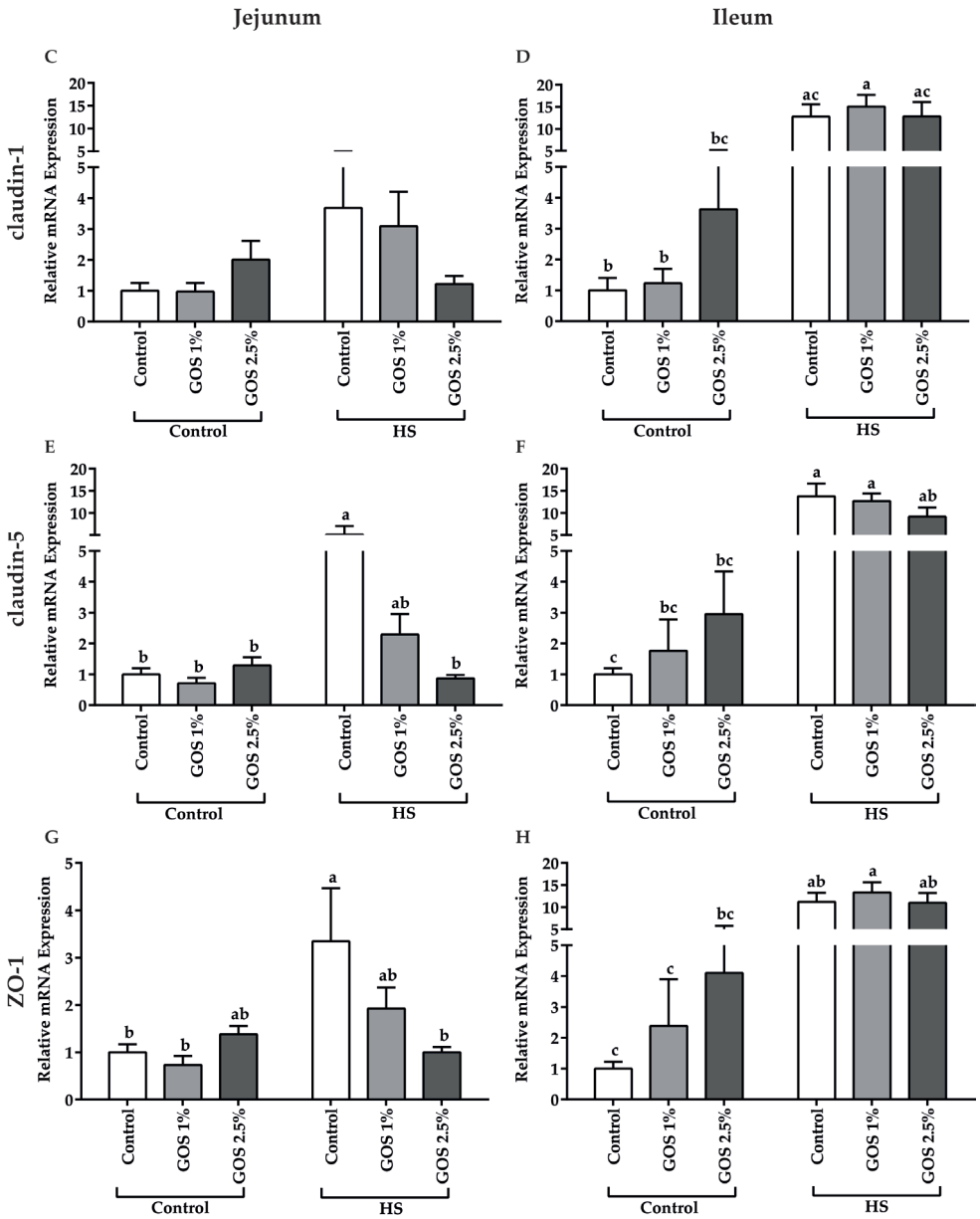

Figure 3. The effect of HS exposure on the mRNA expression of AJ and TJ in jejunum and ileum of chickens fed a control or GOS diet. Chickens fed a control or GOS (1 or 2.5%) diet for 6 days before being exposed to either control or HS conditions for 5 days. mRNA expression of E-cadherin (A,B), claudin-1 (C,D), claudin-5 (E,F) and ZO-1 (G,H) in jejunum (A,C,E,G) and ileum (B,D,F,H) were evaluated by qRT-PCR. Results are expressed as relative mRNA expression (fold of control, normalized to β-Actin) as mean ± SEM, n=6-10 animals experimental group. Different lower-case letters denote significant differences among groups.

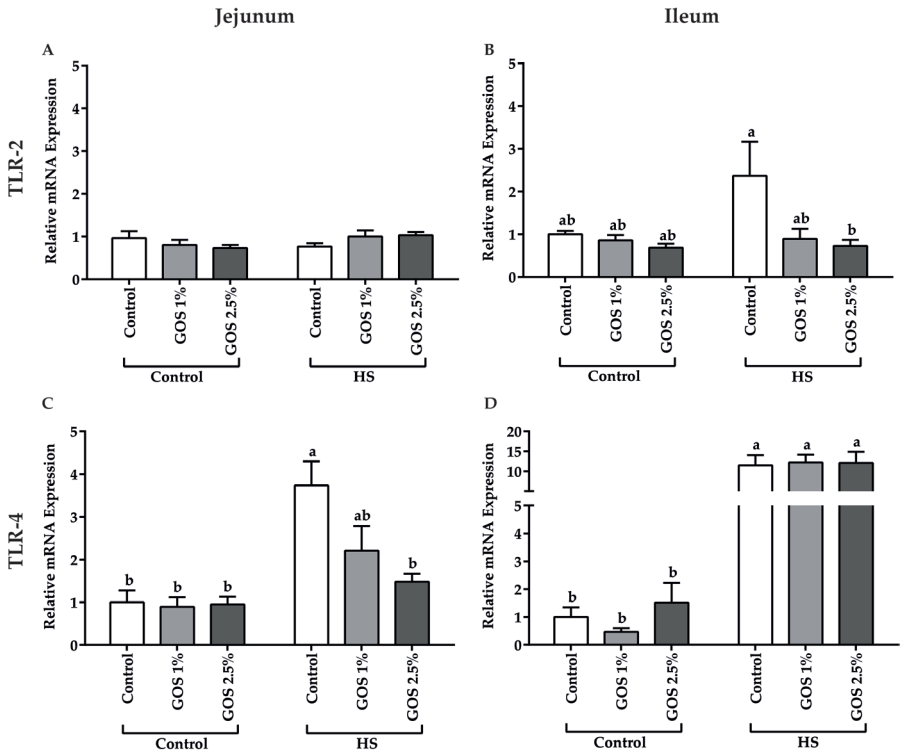

Figure 4. The effect of HS exposure on the mRNA expression of TLR-2 and TLR-4 in jejunum and ileum of chickens fed a control or GOS diet. Chickens fed a control or GOS (1 or 2.5%) diet for 6 days before being exposed to either control or HS conditions for 5 days. mRNA expression of TLR-2 (A,B) and TLR-4 (C,D) in jejunum (A,C) and ileum (B,D) were evaluated by qRT-PCR. Results are presented as relative mRNA expression (fold of control, normalized to β-Actin) as mean ± SEM, n=6-10 animals/experimental group. Different lower-case letters denote significant differences among groups

The upregulation of IL-6 and IL-8 mRNA expression in jejunum and ileum by HS is mitigated by GOS in jejunum

The mRNA expression of the inflammatory markers, IL-6 (Figs. 5A and 5B) and IL-8 (Figs. 5C and 5D), increased significantly in response to HS in both jejunum and ileum, while this effect was more obvious in chicken ileum. Only in the jejunum of heat-exposed animals, a preventive effect on these increased cytokine mRNA levels was observed after feeding the GOS diet (Figs. 5A and 5C).

The decline in IL-8 plasma levels after HS exposure can be prevented by dietary GOS

In chickens exposed to HS, the IL-8 plasma levels were markedly decreased in comparison with control animals (Fig. 5F), while no significant decrease in IL-6 secretion was detected (P=0.31) (Fig. 5E). The plasma levels of heat-exposed

chickens fed a GOS diet, did contain more IL-8 compared to heat-exposed animals fed a control diet (Fig. 5F).

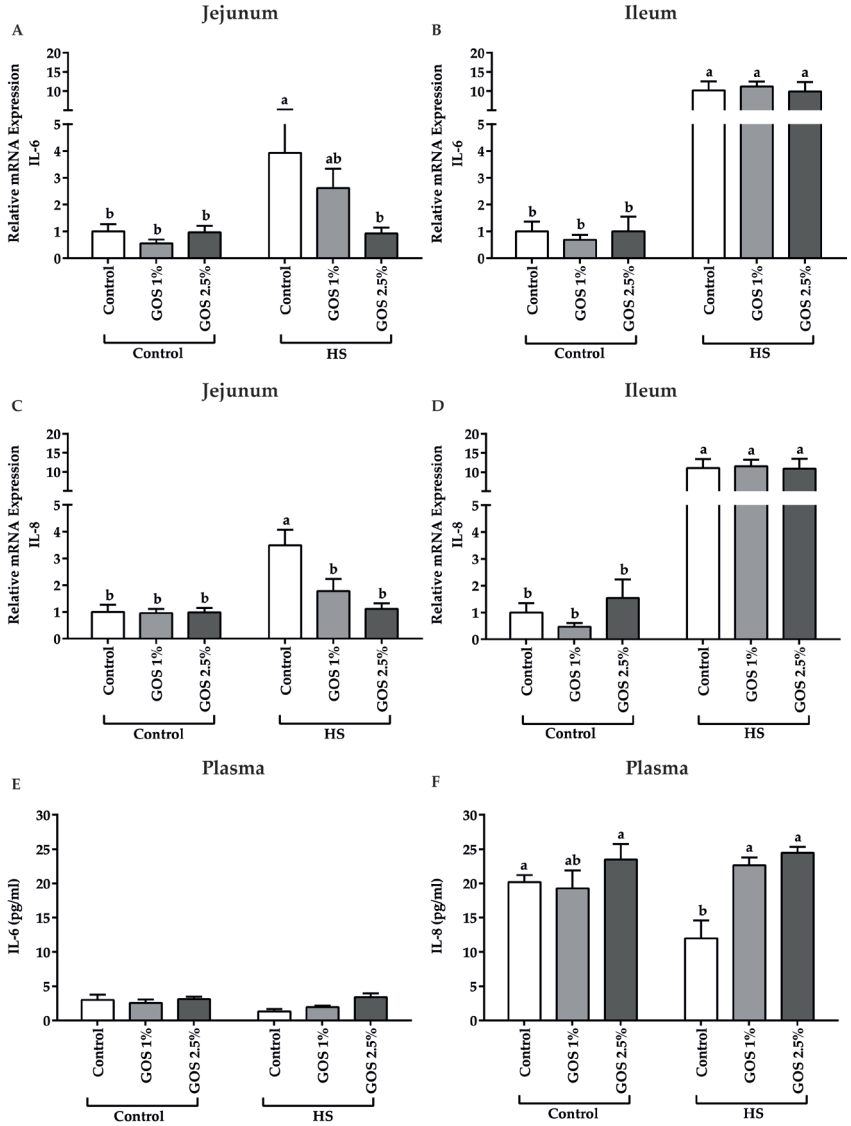

Figure 5. The effect of HS exposure on the IL-6 and IL-8 plasma levels and mRNA expression in jejunum and ileum of chickens fed a control or GOS diet. Chickens fed a control or GOS (1 or 2.5%) diet for 6 days before being exposed to either control or HS conditions for 5 days. mRNA expression of IL-6 (A,B) and IL-8 (C,D) in jejunum (A,C) and ileum (B,D) evaluated by qRT-PCR. Results are expressed as relative mRNA expression (fold of control, normalized to β-Actin) as mean ± SEM. IL-6 (E) and IL-8 (F) secretion in plasma determined by ELISA. Results are expressed in pg/ml as mean ± SEM. n=6-10 animals/experimental group. Different lowercase letters denote significant differences among groups.

HS increases the HIF-1α mRNA levels in ileum

No remarkable changes were observed in the mRNA expression of the oxidative stress markers, haem oxygenase-1 (HO-1) and hypoxia inducible factor, subunit alpha (HIF-1α) in chicken jejunum after HS exposure (Figs. 6A and 6C). In ileum, HIF-1α mRNA levels increased significantly after 5 days HS, while no effect of HO-1 mRNA expression was detected (Figs. 6B and 6D). This increase in HIF-1α mRNA expression in ileum was not attenuated by GOS (Fig. 6D).

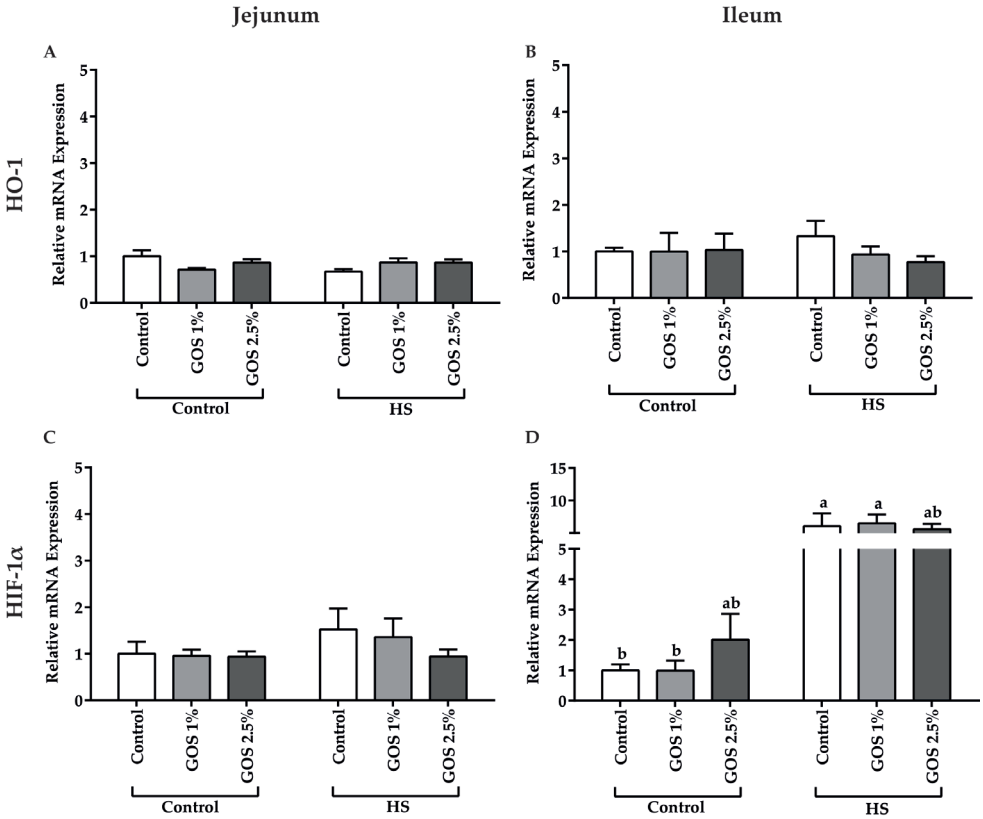

Figure 6. The effect of HS exposure on the mRNA expression of HO-1 and HIF-1α in jejunum and ileum of chickens fed a control or GOS diet. Chickens fed a control or GOS (1 or 2.5%) diet for 6 days before being exposed to either control or HS conditions for 5 days. The mRNA expression of HO-1 (A,B) and HIF-1α (C,D) in jejunum (A,C) and ileum (B,D) measured by qRT-PCR. Results are expressed as relative mRNA expression (fold of control, normalized to β-Actin) as mean ± SEM, n=6-10 animals/experimental group. Different lower-case letters denote significant differences among groups.

Discussion

High ambient temperatures can potentially induce pathophysiological alterations in humans and animals [29]. In particular, the gastrointestinal tract is considered as one of the main target organs affected by HS. The multifaceted response to HS is initiated by HSFs, regulating the expression of HSPs [30,31]. In this study, the gene expression of HSF3, an avian-specific member of the HSF family, was significantly increased after heat exposure in chicken jejunum and ileum, while the HSF1 mRNA levels were upregulated in the ileum. Previous investigations in chicken embryo fibroblasts described that in addition to differences in DNA binding capacity, HSF1 and HSF3, also have different threshold temperatures for activation upon heat exposure [32]. More recently, Xie *et al.* [30] described a tissue specific pattern of heat-induced HSF1 and HSF3 expression, which may be related to the level of oxidative damage. In consideration of these findings, we analysed the mRNA expression levels of the oxidative stress markers, HO-1 and HIF-1α, which are known to play a crucial role in response to heat-induced oxidative stress and heat acclimation [33–35] and are characterized in chicken intestines [36]. A significant increase in HIF-1α mRNA levels was observed only in the ileum of heat-exposed chickens compared to control chickens.

Upregulated and activated HSFs target the major heat inducible proteins such as HSP70 and HSP90 [37], which occupy a central role in the regulation of protein homeostasis during physiological and pathological conditions [38], and are currently considered as general markers of tissue injury [39,40]. The chickens subjected to HS showed a significant upregulation of HSP70 and HSP90 in mRNA levels in both jejunum and ileum, while the corresponding protein expression was only increased significantly for HSP70, in jejunum as well as in the ileum. HSP70 plays a major role in the adaptive response to HS in broilers by improving the antioxidant capacity, inhibiting lipid peroxidation and increasing the activity of digestive enzyme activity [41,42]. In contrast to the results in jejunum and ileum, no HS-related alterations in HSP70 and HSP90 mRNA expression were observed in duodenum and colon, indicating again differences in the susceptibility of the individual parts of the intestines. These findings are in line with a study Zhang *et al.* [43] in which differences in the mRNA expression of HSF3 and HSP70 between two chicken breeds and tissue-specific differences during heat treatment are described.

Another important adaptive response of the body to HS is increasing the peripheral blood flow, in turn resulting in a reduced blood supply in the intestines, and a hypoxia-induced oxidative stress response. Moreover, the gut epithelial barrier becomes ischemic, leading to a dysfunctions of AJ and TJ and loss of barrier integrity [44]. In this study, only in chicken jejunum, a significant increase in E-cadherin mRNA levels was detected after heat exposure, which could be regarded as a compensatory response related to the observed decrease in cadherins protein expression. Lang *et al.* demonstrated that the E-cadherin gene and protein expression

has been decreased in heat-exposed human lung adenocarcinoma cells [45]. The link between E-cadherin and HSP expression was recently confirmed by Chen *et al.* observing a downregulation of E-cadherin protein levels in colorectal cancer cells directly exposed to rHSP90α [46]. The *in vivo* data with respect to E-cadherin is in agreement with our recently published *in vitro* results with Caco-2 cells exposed to HS [47]. In addition to E-cadherin, heat exposure induced alterations in the level of mRNA expression of the TJ proteins claudin-5 and ZO-1, which was significantly increased in jejunum and ileum, and claudin-1 was upregulated in the ileum. The occludin mRNA levels remained unaffected after HS, which is in line with our previous results showing that in a Caco-2 cell model, the expression of TJ proteins remained unaffected by HS [47]. The absence of the microbiota in this Caco-2 *in vitro* model, that might play a role in the regulation of TJ permeability during HS [48], could be a possible explanation for the discrepancies between the *in vitro* and *in vivo* findings. A study in pigs investigated the effect of HS on the protein expression of TJs in the ileum and showed an increase in claudin-3 and occludin protein expression in the heat-exposed animals, while no differences in claudin-1 expression were observed [29].

Disruption of these junctional proteins may lead to increased permeability of the intestinal barrier to luminal antigens, which will initiate inflammatory signalling via TLRs [12,13]. Although in this study, no significant changes were observed in TLR-2 mRNA expression along the intestine after heat exposure, the TLR-4 mRNA expression was significantly upregulated in both chicken jejunum and ileum. This TLR-4 upregulation could be induced by the invasion of Gram-negative bacteria due to the disrupted intestinal barrier [49] or by a direct effect of the heat exposure, since TLR-4 has been described as a stress-related biosensor in initial injury responses {reviewed in [14]}. Furthermore, TLR-4 activation can contribute to the intestinal barrier breakdown, since it has been demonstrated that TLR-4 knockout mice were protected from burn-induced intestinal hyperpermeability [13].

The HS-induced changes in HSPs, HIF-1α, junctional proteins and TLR-4 expression levels, were accompanied with an inflammatory reaction in the intestine, since chickens exposed to HS, showed significantly higher mRNA expression level of IL-6 and IL-8 in both jejunum and ileum, whereas the production of these cytokines in jejunum and ileum remained unchanged (Fig. S3). Independent of the local effects on IL-6 and IL-8 mRNA levels under HS conditions, probably caused by the intestinal barrier disruption, penetration of pathogens and exaggerating the extent of TLR-4 signalling, a decrease in IL-8 plasma levels was observed after HS exposure. This could be related to the anti-inflammatory effects of HSPs within a HSR [20,50], as HSPs are activators of anti-inflammatory regulatory T cells [51] and HSP induction most probably blocks the NF-κB activation by stabilizing IκBα [21]. In addition, it is known that HSFs can also display anti-inflammatory properties [52].

Overall, the increased mRNA expression levels of the HSFs, HSPs, TJ proteins,

TLR-4, IL-6 and IL-8 were more pronounced in ileum of heat-exposed chickens compared to the jejunum, suggesting the higher susceptibility of the ileum to heat-induced oxidative stress as the mRNA expression of the oxidative stress marker, HIF-1α, was only upregulated in ileum. Although HS-induced injury has been previously described in the chicken small intestine [7,53], no attention was paid to the differences in susceptibility of individual intestinal segments. However, Santos *et al.* showed the influence of HS on morphological parameters of the duodenal, jejunal and ileal mucosa [54].

A possible explanation for the more severe heat-induced damage observed in ileum could be related to the difference in the microbiota composition between the ileum and jejunum [55,56] and the related anti-inflammatory effects of the microbial HSPs and their related peptides [51,57].

In this study we hypothesized that dietary GOS, supporting the gut homeostasis, may exert protective effects against the HS-induced multifaceted response in the chicken intestine. In the chicken jejunum, all HS-induced changes (HSFs, HSPs, E-cadherin, TJ proteins, TLR-4, IL-6 and IL-8) were prevented by the GOS supplemented diet, resulting in a less severe HSR. However, GOS supplementation failed to modulate the heat-induced changes in the chicken ileum.

Since GOS has been associated with stimulating the beneficial microbial population [26,58], the protective effect of GOS after HS could be related to changes in microbial HSPs induced by GOS. However, GOS could also prevent the heat-induced upregulation of HSP70 and HSP90 in the Caco-2 monolayer lacking the microbial environment [47]. The latter findings suggest that GOS has also direct effects on the expression of TJ proteins [19]. Microbiota-independent mechanisms by direct interaction on immune and epithelial cells have been confirmed recently [59–61]. Moreover, the observed effects of GOS might be similar to the macromolecule-stabilizing character of some sugars like trehalose-based oligosaccharides, that act as antioxidants [62,63]. From different *in vivo* studies it is known that supplementation of the diet with non-digestible oligosaccharides like GOS and mannan-oligosaccharides (MOS) improved the intestinal integrity [28,64] and exert direct immuno-modulatory effects {reviewed for GOS/ fructo-oligosaccharides by Jeurink *et al.* 2013 [65]}. In chickens, an improvement of the intestinal mucosal architecture as observed in a study with dietary supplementation of MOS and probiotics mixtures [66] and fructo-oligosaccharides, inulin and MOS are also described to reduce the susceptibility of chicken intestine to colonization by pathogenic bacteria, like *Salmonella* spp. [67,68]. Further investigations should aim to understand the possible direct and indirect mechanisms underlying the integrity- and immune-regulating effects of GOS associated to intestinal injury.

Conclusions

Our results provide for the first time evidence for a difference in susceptibility of individual intestinal segments to HS as demonstrated by the assessment of different biomarkers including HSPs, HSF, AJs and TJs, cytokines and oxidative stress markers. Alterations in the level of expression of these biomarkers was more pronounced in ileum of heat-exposed chickens compared to the jejunum. Dietary application of GOS, an oligosaccharide that acts as a prebiotic, but also exerts direct, microbiota-independent effects and stabilizes intestinal integrity, could not mitigate the alterations in the ileum, but successfully prevented all HS-induced changes (HSFs, HSPs, E-cadherin, TJ proteins, TLR-4, IL-6 and IL-8) in the jejunum.

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Supporting Information

Table S1. Feed composition standard broiler diet

Ingredients	Value (g/kg)	Nutrient	Value (g/kg)	Amino Acids	Value (g/kg)
Maize	322,20	Dry matter	879,60	Isoleucine	8,30
Wheat + xylanase	250,00	Crude ash	50,10	Leucine	15,93
Soya	225,00	Crude protein	204,20	Lysine	11,75
Rapeseed meal	30,00	Crude fat	70,80	Methionine	5,19
Peas (dry)	50,00	Crude fibre	30,50	Cysteine	3,44
Soy bean (heated)	50,00	Carbohydrates	525,30	Phenylalanine	9,67
Lard	20,00	Organic matter	146,00	Tyrosine	6,95
Soy bean oil	20,00	Starch	389,20	Threonine	7,96
Premix (maize)	5,00	Sugars	40,40	Tryptophan	2,33
Chalk	13,00	Neutral detergent fibre	99,30	Valine	9,34
Limestone	0,00	Acid detergent fibre	39,70	Arginine	13,14
Monocalcium phosphate	6,00	Calcium	7,90	Histidine	5,29
Salt (NaCl)	2,20	Total phosphorus	4,90	Alanine	9,33
$NaHCO_3$	2,00	Magnesium	1,60	Aspartic acid	19,42
Phytase	0,10	Potassium	9,00	Glutamic acid	38,27
L-lysine HCL	1,70	Sodium	1,48	Glycine	8,39
DL-methionine	2,20	Chlorine	2,00	Proline	12,33
L-threonine	0,60	Base-excess (mEq/kg)	235,50	Serine	9,85
		Net energy (MJ/kg)	10,40		
		Linoleic acid (MJ/kg)	26,80		
		Fe (mg/kg)	136,00		
		Mn (mg/kg)	21,00		
		Zn (mg/kg)	30,00		
		Cu (mg/kg)	6,00		

Table S2. Primer sequences used for qRT-PCR

Genes	Primer sequence (5′-3′)		AT	References
	Forward	Reverse		
β-Actin	ATGTGGATCAGCAAGCAGGAGTA	TTTATGCGCATTTATGGGTTTTGT	61	NM_205518.1
claudin-1	CTGATTGCTTCCAACCAG	CAGGTCAAACAGAGGTACAGG	58	NM_001013611
claudin-5	CATCACTTCTCCTTCGTCAGC	GCACAAAGATCTCCCAGGTC	58	NM_204201
E-cadherin	GACAGGGACATGAGGCAGAA	GCCGTGACAATGCCATTCTC	64.3	NM_001039258.2
HIF-1α	ACCATTACCATACTTCAGCAG	CTTCACATCATCCAGACGTTC	65	NM_204297
HO-1	CTTCGCACAAGGAGTGTTAAC	CATCCTGCTTGTCCTCTCAC	63	NM_205344
HSF1	CAGGGAAGCAGTTGGTTCACTACACG	CCTTGGGTTTGGGTTGCTCAGTC	65	L06098.1
HSF3	TCCACCTCTCCTCTCGGAAG	CAACAGGACTGAGGAGCAGG	57	L06126.1
HSP70	TCTCATCAAGCGTAACACCAC	TCTCACCTTCATACACCTGGAC	55	JX827254.1
HSP90	ATGCCGGAAGCTGTGCAAACACAGGACCAA	GGAATCAGGTTAATTTTCAGGTCTTTTCCA	63.1	NM_001109785.1
IL-6	GCTCGCCGGCTTCGA	GGTAGGTCTGAAAGGCGAACAG	58.7	HM179640.1
IL-8	CACGTTCAGCGATTGAACTC	GACTTCCACATTCTTGCAGTG	61.2	NM_205018.1
occludin	ACGGCAGCACCTACCTCAA	GGGCGAAGAAGCAGATGAG	61.2	D21837.1
TLR-2	CCTGCAACGGTCACTTCAG	GTCTCAGGGCTTGTTCTTCAG	59	NM_204278
TLR-4	CTGACCTACCCATCGGACAC	GCCTGAGAGAGGTCAGGTTG	59	NM_001030693
ZO-1	CTTCAGGTGTTTCTCTTCCTCCTC	CTGTGGTTTCATGGCTGGATC	59	XM_413773

AT, Annealing temperature (°C)

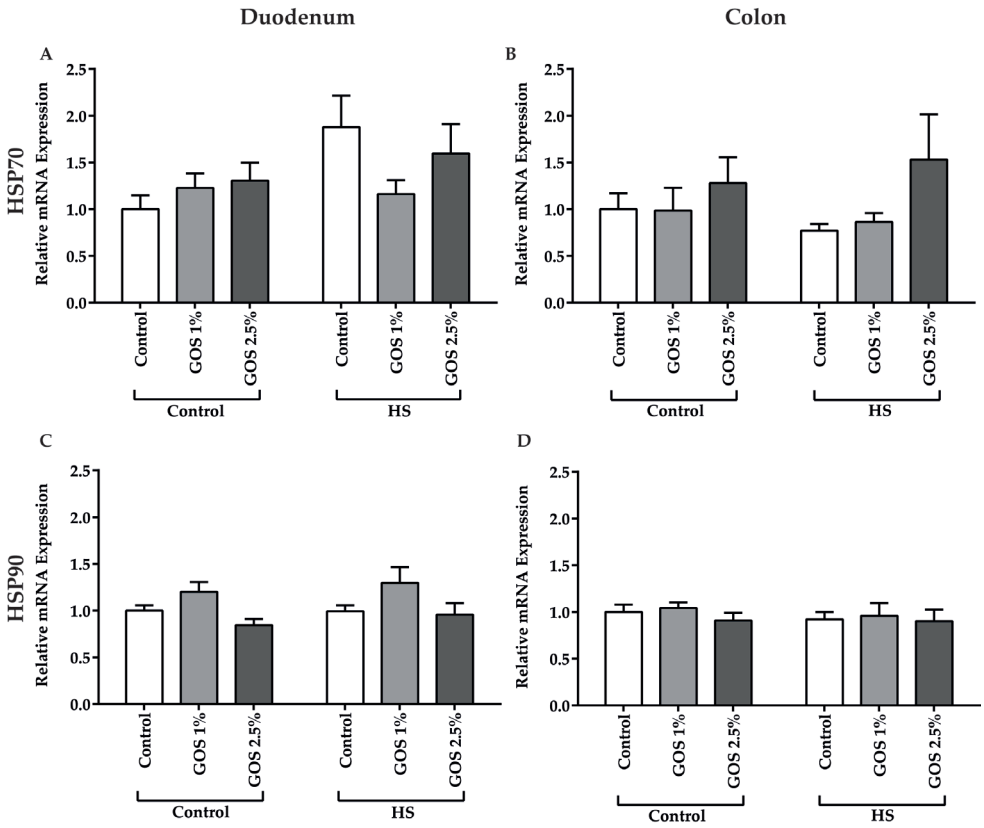

Figure S1. The effect of heat stress exposure on the mRNA expression of HSPs in duodenum and colon of chickens fed a control or GOS diet. Chickens fed a control or GOS 2.5% diet for 6 days before being exposed to control or heat stress conditions for 5 days. The mRNA expression of HSP70 and HSP90 was quantified in duodenum (A,C) and colon (B,D) by qRT-PCR. Results are expressed as relative mRNA expression (fold of control, normalized to β-Actin) as mean ± SEM, n=10 animals/experiment group. Different lower-case letters denote significant differences among groups.

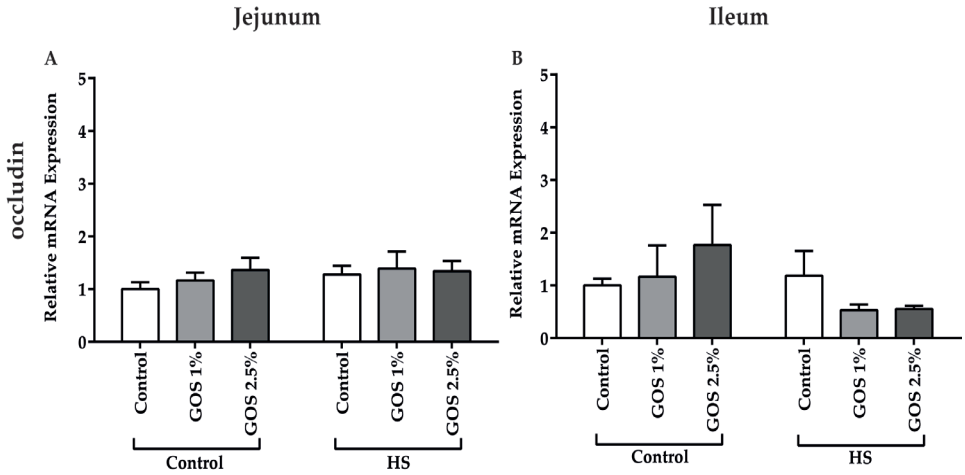

Figure S2. The effect of heat stress exposure on the mRNA expression of occludin in jejunum and ileum of chickens fed a control or GOS diet. Chickens fed a control or GOS 2.5% diet for 6 days before being exposed to control or heat stress conditions for 5 days. The mRNA expression of occludin was quantified in jejunum (A) and ileum (B) by qRT-PCR. Results are expressed as relative mRNA expression (fold of control, normalized to β-Actin) as mean ± SEM, n=6-10 animals/experiment group. Different lower-case letters denote significant differences among groups.

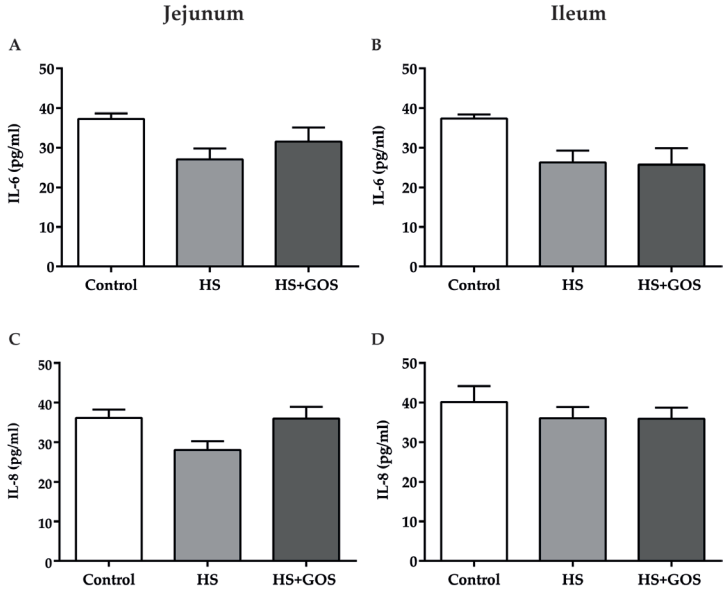

Figure S3. The effect of heat stress exposure on IL-6 and IL-8 production in jejunum and ileum of chickens fed a control or GOS diet. Chickens fed a control or GOS 2.5% diet for 6 days before being exposed to control or heat stress conditions for 5 days. ELISA was performed for quantification of IL-6 and IL-8 production in jejunum (A,C) and ileum (B,D) homogenates. Results are expressed in pg/ml as mean ± SEM, n=5 animals/experimental group.

Chapter 5

Heat stress alters the gene expression of nutrient transporters along the chicken small intestine and dietary galacto-oligosaccharides are able to modulate this response

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Abstract

One of the primary functions of the intestinal epithelium is the transport of fluids, nutrients and electrolytes from and to the intestinal lumen. These transport processes are facilitated by different membrane transporters and these nutrient transporters may be altered under stress conditions. Hence, this study aimed to investigate the effect of heat stress (HS) on the expression of intestinal brush border nutrient transporters, including intestinal peptide transporter (PepT-1), glutamate/aspartate amino acid transporter-3 (EAAT-3), sugar transporters, such as the fructose transporter (GLUT-5) and the sodium-dependent glucose transporter 1 (SGLT-1) in jejunum and ileum of broiler chickens. In addition, the expression of the liver-expressed antimicrobial peptide (LEAP-2), as a representative of the antimicrobial peptides that play a key role in the innate host defence mechanism in the chicken intestines, was measured. All these parameters were determined in chickens under control and HS conditions with or without dietary galacto-oligosaccharides (GOS) in their diet. To this end, chickens were fed a control diet or diet supplemented with 1% or 2.5% GOS for 6 days prior to and during a HS challenge for 5 days (38–39°C, 8h per day). mRNA levels of PepT-1, EAAT-3, GLUT-5, SGLT-1 and LEAP-2 were examined in jejunum and ileum by qRT-PCR. Results showed that the mRNA levels of PepT-1 and GLUT-5 were significantly upregulated in jejunum and ileum of HS-exposed chickens. In contrast, LEAP-2 mRNA levels were decreased under HS conditions. Feed supplementation with GOS significantly attenuated the HS-induced upregulation of PepT-1 and GLUT-5 in chicken jejunum and prevented the HS-induced downregulation of LEAP-2 mRNA expression. In conclusion, the presented results demonstrate that HS selectively modifies the expression of nutrient transporters along the chicken small intestine and dietary application of GOS mitigates these HS-induced alterations in nutrient transporters in the jejunum.

Introduction

The transport of nutrients and other dietary substances from the intestinal lumen to the portal circulation is considered as a primary function of the intestinal epithelium. In the gastrointestinal tract, the transporters responsible for the nutrient absorption from the lumen are mainly located in brush border membranes of villus cells and upper crypt cells [1]. These nutrient transporters regulate the uptake of nutrients, including products of protein and carbohydrate digestion, such as amino acids, peptides, glucose and galactose [2]. Familiar examples of these intestinal brush border nutrient transporters are the peptide transporter (PepT-1), the glutamate/aspartate amino acid transporter (EAAT-3), the sugar transporters, like fructose transporter (GLUT-5) and sodium-dependent glucose transporter 1 (SGLT-1) [3,4].
A growing body of evidence shows that the function of nutrient transporters may be altered under stress and pathophysiological conditions and these changes may contribute to the pathophysiology of intestinal inflammatory diseases [5,6]. Heat stress (HS) is one of the most relevant environmental stressors, and exposure to HS can lead to multi-organ injury in both humans and animals [7,8]. Induction of HS is particularly associated with intestinal mucosal injury, leading to disruption of intestinal integrity and inflammatory reactions [9,10].
Modern poultry are considered to be one of the most vulnerable animal species to the adverse effects of HS, including gut-associated oxidative stress, inflammatory responses and intestinal barrier disruption, which are related to their rapid growth rate and high metabolic activity [11,12].
We have previously shown that feed supplementation with non-digestible oligosaccharides, specifically galacto-oligosaccharides (GOS), effectively regulates the immunologic homeostasis and preserves the epithelial integrity in the chicken jejunum affected by HS [10].
In this study we aimed to investigate the effect of HS induction on the expression of different brush border membrane nutrient transporters, including PepT-1, EAAT-3, GLUT-5 and SGLT-1 in jejunum and ileum of HS-exposed chickens. The effect of HS was also examined on the expression of LEAP-2 (liver-expressed antimicrobial peptide-2), which has been recognized as key mediator of the innate host defence mechanisms in small intestinal tissues of chickens and can serve as a measure for intestinal immune homeostasis [13]. Furthermore, the effect of dietary GOS on the expression of nutrient transporters and LEAP-2 in the small intestine of these HS-exposed chickens was studied.

Materials and Methods

Animals and experimental design

The experimental protocol was approved by the animal welfare committee of the University of Veterinary Medicine Hannover according to BGBl. I S. 1105/2 (in line with DIRECTIVE 2010/63/EU). The present data were obtained from further analyses of tissue samples from the *in vivo* experiment we published recently [10]. Briefly, this study was conducted with sixty 15-day-old Ross broilers, which were randomly divided into 6 groups (control group, control group + 1% GOS, control group + 2.5% GOS, HS group, HS group + 1% GOS, HS group + 2.5% GOS). The chickens were housed in two environmentally controlled rooms (control and HS) with 3 special bird units equipped with a lighting program of 16h light and 8h dark per day. Birds were acclimatized for 6 days at a temperature of 22-26°C. Thereafter, the control groups were housed under thermal neutral conditions at a temperature of 22-23°C, while the room temperature for the HS-exposed groups was 38-39°C for 8h during the daylight period for 5 consecutive days, and 22-23°C during the remaining period. Water and feed were provided *ad libitum* during the entire experimental period. The broilers were fed either a standard broiler diet (Table S1) or the standard diet supplemented with 1% or 2.5% GOS (Research Diet Services, Wijk-bij-Duurstede, The Netherlands). GOS were obtained from FrieslandCampina Domo (Vivinal® GOS syrup, Borculo, The Netherlands) containing oligosaccharides with a degree of polymerisation (dp) of 2-8 with approximately 59% (w/w) galacto-oligosaccharides, 21% (w/w) lactose, 19% (w/w) glucose and 1% (w/w) galactose on dry matter (dry matter of 75%).

Sample collection

At the end of the experimental period, the animals were sacrificed by cervical dislocation and samples from jejunum (5 cm before Meckel's diverticulum) and ileum (5 cm before ileo-cecal transition) were collected, directly rinsed in phosphate buffer saline (PBS), snap frozen in liquid nitrogen and stored at -80°C for qRT-PCR analysis.

Quantitative RT-PCR (qRT-PCR) analysis

Tissue samples from jejunum and ileum suspended in RNA lysis buffer containing β-mercaptoethanol were homogenized using the TissueLyser (Qiagen, Hilden, Germany) and total RNA was isolated using spin columns based on manufacturer's instructions (Promega, Madison, WI, USA). Subsequently, RNA was reverse-transcribed to cDNA using iScriptTM cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The PCR reaction mixture, containing SYBR Green Supermix (Bio-Rad), was prepared based on manufacturer's instructions. qRT-PCR analysis was performed using the CFX96™ Real-Time system (Bio-Rad) and the values were determined using CFX96 Real-Time PCR software (Bio-Rad). Commercially manufactured gene

specific primers (Eurogentec, Seraing, Belgium) were used after confirmation of specificity and efficiency tests by qRT-PCR with dilution series of pooled cDNA at a temperature gradient (55°C to 65°C) for primer-annealing and subsequent melting curve analysis (Table 1). The mRNA quantity was calculated relative to the expression of β-Actin used as reference gene.

Table 1. Primer sequences used for qRT-PCR

	Primer sequence (5′-3′)			
Genes	**Forward**	**Reverse**	**AT**	**References**
β-Actin	ATGTGGATCAGCAAGCAGGAGTA	TTTATGCGCATTTATGGGTTTTGT	61	NM_205518.1
EAAT-3	TGCTGCTTTGGATTCCAGTGT	AGCAATGACTGTAGTGCAGAAGTAATATATG	55	XM_424930
GLUT-5	TTGCTGGCTTTGGGTTGTG	GGAGGTTGAGGGCCAAAGTC	59	XM_417596
LEAP-2	CTCAGCCAGGTGTACTGTGCTT	CGTCATCCGCTTCAGTCTCA	65	NM_001001606.1
PepT-1	CATCACTTCTCCTTCGTCAGC	GCACAAAGATCTCCCAGGTC	58.7	AY029615.1
SGLT-1	TGTCTCTCTGGCAAGAACATGTC	GGGCAAGAGCTTCAGGTATCC	63.1	AJ236903.1

AT, Annealing temperature (°C)

5

Statistical analysis

Experimental results are expressed as mean ± SEM of n=6-10 animals/experiment. Statistical analyses were performed using GraphPad Prism (version 6.05) (GraphPad, La Jolla, CA, USA). Differences between groups were statistically determined by using a two-way ANOVA test with Bonferroni post-hoc test. $P < 0.05$ were considered statistically significant. Different lower-case letters denote significant differences among groups.

Results

HS-induced upregulation of PepT-1 is attenuated by GOS supplementation, whereas EAAT-3 remains unaffected

HS exposure resulted in a significant upregulation of the mRNA levels of the peptide transporter PepT-1 in jejunum and ileum (Figs. 1A and 1B). Although feed supplementation with GOS attenuated the HS-induced upregulation of PepT-1 in both jejunum and ileum (Figs. 1A and 1B), only the jejunum showed a statistically significant effect (Fig. 1A). In addition, no significant effect of HS exposure or type of diet was observed on the mRNA expression of the amino acid transporter EAAT-3 in jejunum and ileum (Figs. 1C and 1D).

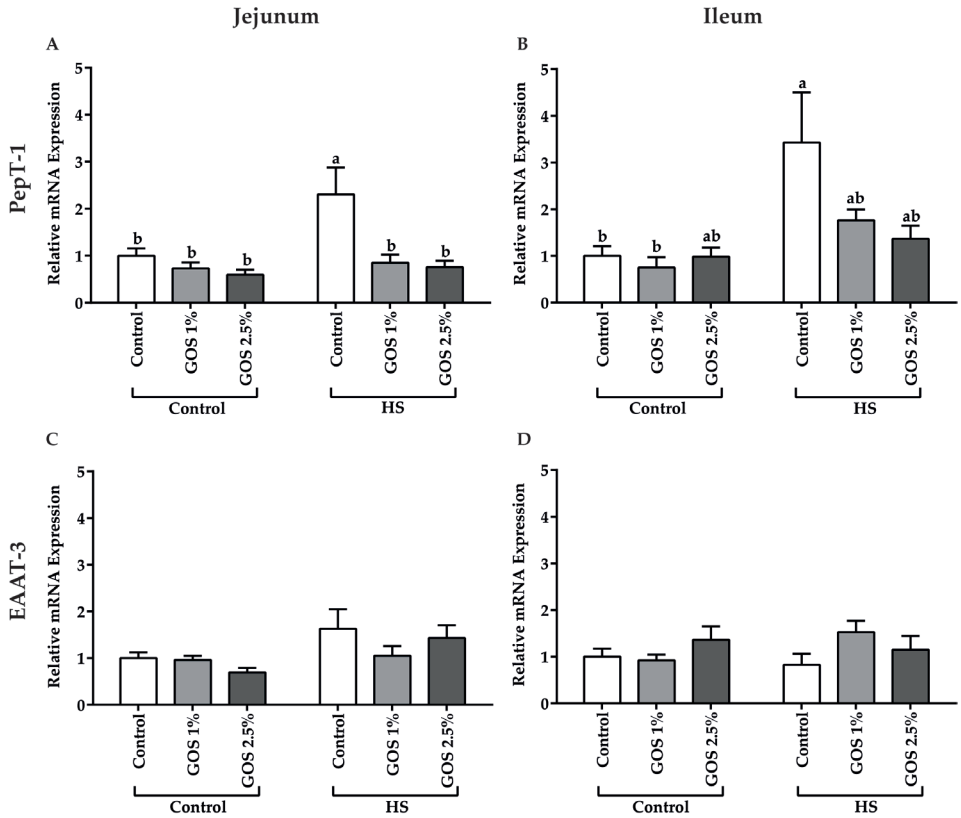

Figure 1. The effect of HS exposure on the mRNA expression of PepT-1 and EAAT-3 in jejunum and ileum of control or GOS-fed chickens. Chickens fed a control or GOS-supplemented (1 or 2.5%) diet for 6 days prior to exposure to control or HS conditions for 5 days. mRNA expression of PepT-1 (A,B) and EAAT-3 (C,D) were evaluated in jejunum (A,C) and ileum (B,D) by qRT-PCR. Results are expressed as relative mRNA expression (fold of control, normalized to β-Actin) as mean ± SEM, n=6-10 animals/experimental group. Different lower-case letters denote significant differences among groups.

HS-induced upregulation of GLUT-5 is attenuated by GOS supplementation in jejunum, whereas SGLT-1 remains unaffected

HS exposure resulted in a significant increase in mRNA expression of the sugar transporter GLUT-5 in jejunum and ileum (Figs. 2A and 2B). GOS supplementation significantly attenuated the HS-induced increase in GLUT-5 mRNA expression in jejunum (Fig. 2A). However, in ileum the HS-induced GLUT-5 mRNA expression was not changed in GOS-fed chickens compared to chickens fed a control diet (Fig. 2B). No significant effect of HS exposure or GOS supplementation was observed on the mRNA expression of the sugar transporter SGLT-1 in jejunum or ileum (Figs. 2C and 2D).

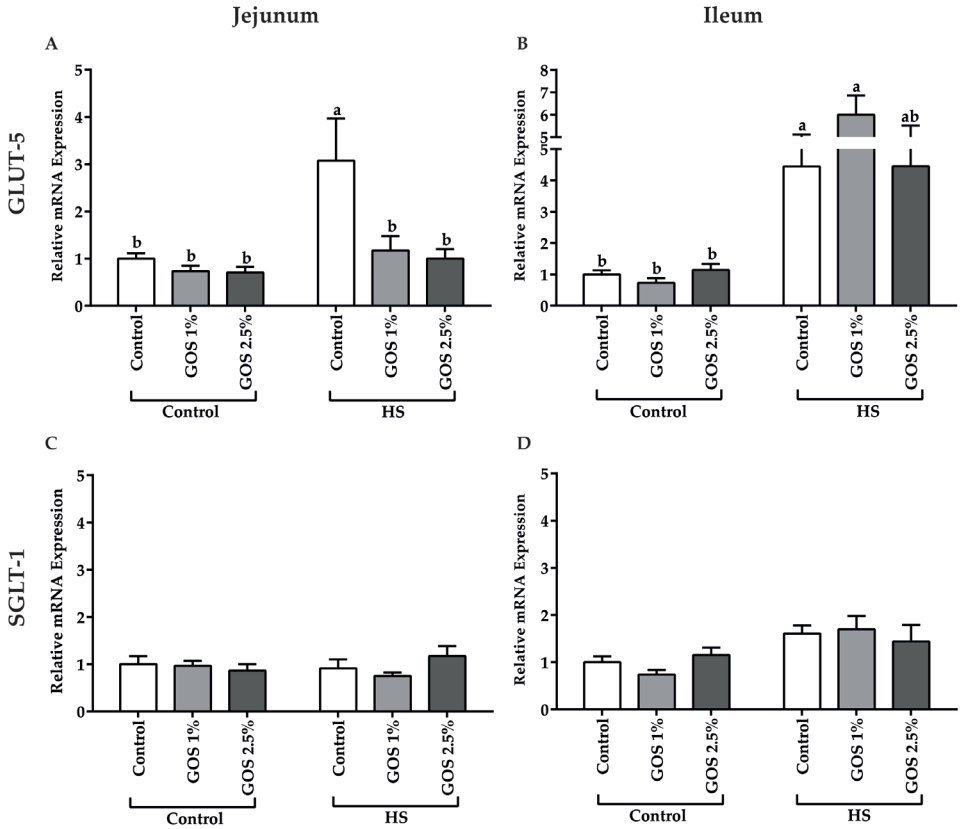

Figure 2. The effect of HS exposure on the mRNA expression of GLUT-5 and SGLT-1 in jejunum and ileum of control or GOS-fed chickens. Chickens fed a control or GOS-supplemented (1 or 2.5%) diet for 6 days prior to exposure to control or HS conditions for 5 days. mRNA expression of GLUT-5 (A,B) and SGLT-1 (C,D) were evaluated in jejunum (A,C) and ileum (B,D) by qRT-PCR. Results are expressed as relative mRNA expression (fold of control, normalized to β-Actin) as mean ± SEM, n=6-10 animals/experimental group. Different lower-case letters denote significant differences among groups.

HS exposure downregulates the LEAP-2 expression in ileum

HS exposure downregulated the mRNA expression of the antimicrobial peptide LEAP-2 in ileum (Fig. 3B). However, this decrease in LEAP-2 under HS conditions was not significantly different in jejunum (Fig. 3A). GOS supplementation had a tendency (no significant effect) to prevent the HS-induced downregulation of LEAP-2 in jejunum and ileum (Figs. 3A and 3B).

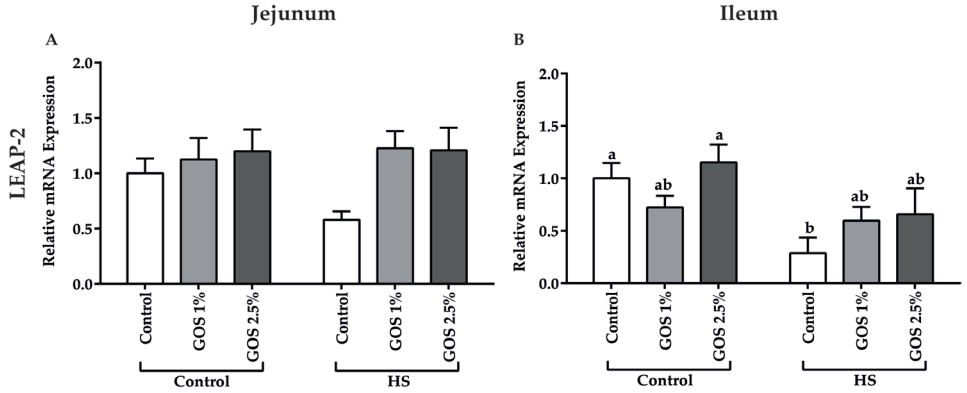

Figure 3. The effect of HS exposure on the mRNA expression of LEAP-2 in jejunum and ileum of control or GOS-fed chickens. Chickens fed a control or GOS-supplemented (1 or 2.5%) diet for 6 days prior to exposure to control or HS conditions for 5 days. mRNA expression of LEAP-2 (A, B) was evaluated in jejunum (A) and ileum (B) by qRT-PCR. Results are expressed as relative mRNA expression (fold of control, normalized to β-Actin) as mean ± SEM, n=6-10 animals/experimental group. Different lower-case letters denote significant differences among groups.

Discussion

Exposure to high ambient temperature is known to affect the intestinal homeostasis, leading to disruption of intestinal integrity, inflammatory responses and alterations in nutrient absorption [14].
In this study we focused on the effect of HS on the expression of different nutrient transporters, including amino acid/peptide transporters and sugar transporters in jejunum and ileum of broiler chickens. Our results showed that the PepT-1 mRNA expression was significantly upregulated in jejunum and ileum in response to HS, whereas the EAAT-3 mRNA expression remained unchanged after HS exposure. This differential response to HS of the mRNA expression of the free-amino acid transporter EAAT-3 and peptide transporter PepT-1 may be related to their function. While the function of EAAT-3 is limited to the uptake of free amino acids, particularly glutamate [2], the PepT-1 transporter accepts a broader range of peptides as substrates, including almost all di/tripeptides [15,16]. We previously showed that exposure to HS is associated with disruption of intestinal integrity and intestinal inflammation in chickens [10]. *In vitro* and *in vivo* investigations demonstrated that intestinal inflammation increases the expression of PepT-1. This increase in PepT-1 expression may lead to enhanced transport of bacterial products with a peptide structure, such as *N*-formylmethionylleucyl-phenylalanine (fMLP), which will exacerbate the intestinal inflammation [17,18].
As an adaptive response of the body to HS, the blood flow shifts from visceral tissues to the peripheral circulation, resulting in hypoxic conditions and ATP depletion in intestinal epithelium [9,14]. During hypoxic conditions, ATP generation switches from the highly efficient pathway of oxidative phosphorylation, yielding 32 ATPs per glucose molecule, to a less efficient glycolysis pathway, producing only 2 ATPs per glucose molecule [1]. Therefore, we next examined the influence of HS exposure on the expression of the intestinal brush border sugar transporters GLUT-5 and SGLT-1. Although HS did not induce significant alterations in the SGLT-1 mRNA expression, GLUT-5 mRNA levels were significantly upregulated in jejunum and ileum of HS-exposed chickens. One of the most important functional differences between the SGLT-1 and GLUT sugar transporter family is related to their association with ATP usage. SGLT-1 is known to have an ATP-dependent function in the absorption of glucose, while the GLUT transporter family, including GLUT-5, transports sugars in a passive (energy-independent) manner [19]. Walker *et al.* suggested that in conditions where the oxygen and ATP supply is limited, the intestinal epithelium utilizes the energy-independent mechanism of sugar transport [19]. Different studies have indicated that sugar transporters from the GLUT-family, including GLUT-5, are upregulated in response to hypoxic conditions [20]. Furthermore, the GLUT-5 nutrient transporter is mainly involved in the transport of fructose and has a low efficiency of glucose transport compared to sodium-dependent glucose transporter SGLT-1. Related to the low efficiency of glucose transport, it may be

possible that the increased GLUT-5 gene expression leads to a higher GLUT-5 protein expression, thus maximizing the total glucose transport and compensating for the impaired SGLT-1 transporter function [3] during HS conditions associated with intestinal hypoxia and ATP depletion [9,14].

Another adaptive response of the body to HS, is assumed to be related to reduction in food intake to prevent the metabolic heat production [21]. A reduced feed intake results in restricted nutrient absorption, alterations in intestinal morphology and function, which may increase the risk of bacterial sepsis [22]. However, in the current study, no significant change between the average weight gain of the HS-exposed chickens and the chickens under control conditions was observed (data not shown), which can be explained by the limited daily heat exposure time. Clinical observations revealed that during HS, feed intake almost ceased, but during the remaining hours at lower ambient temperatures, the animals did consume enough feed to avoid weight losses.

HS induces intestinal damage, hence in the current study the HS-induced innate immune response in the small intestine was investigated by examining the mRNA expression of the antimicrobial peptide LEAP-2. LEAP-2, which is predominantly expressed in the chicken small intestine [23], is part of the innate host defence mechanisms and its downregulation has been associated with increased susceptibility to infectious intestinal disorders in chickens [24]. Our results showed that LEAP-2 mRNA expression was downregulated in jejunum and ileum of HS-exposed chickens. Although the mechanism by which HS downregulates the LEAP-2 mRNA expression is not yet known, different reports have demonstrated that the decrease in LEAP-2 expression correlates with intestinal injuries and inflammation [24,25]. We previously demonstrated that exposure to HS selectively affects the different intestinal segments in broiler chickens and the most obvious modifications in intestinal integrity and inflammatory responses were observed in the ileum, which is in agreement with the significant downregulation of LEAP-2 in this study [10].

Feed supplementation with GOS has been shown to promote the population of beneficial bacteria in the gut, to enhance the intestinal integrity, to stimulate the intestinal defence mechanism and to attenuate the detrimental effects of diseases and conditions affecting gut health, including HS [10,26,27]. In this study, we showed that dietary GOS could significantly prevent the HS-induced upregulation of the nutrient transporters PepT-1 and GLUT-5 in the jejunum, but not in the ileum. GOS was also more effective in the chicken jejunum compared to the ileum related to the protective effects on HS-induced intestinal inflammation and barrier dysfunction demonstrated in our previous investigations [10]. A possible explanation for the differential effects of GOS along the small intestine could be their modifying effect on the microbiota composition in jejunum and ileum. For instance, the population of *Lactobacillus* spp., which is known to exert the intestinal barrier preserving effects and promote anti-inflammatory mechanisms [28], are more abundant in jejunum

(>99% of microflora population) compared to ileum (~70% of microflora population) [29,30].

Conclusions

This study demonstrates that exposure to HS selectively modifies the expression of nutrient transporters along the small intestine. This may highlight the involvement of nutrient transporters in the pathophysiology of intestinal damage followed by HS exposure. The protective effects of GOS on changes in nutrient transporter expression in the jejunum after HS exposure may be associated with the known effects of GOS, including stimulating the population of beneficial bacteria, promoting intestinal (epithelial) barrier integrity, and attenuating the HS-induced oxidative stress thereby mitigating the anti-inflammatory response. Inflammation and impairment of the intestinal barrier integrity under HS conditions can also influence innate defence mechanisms, such as the expression of antimicrobial peptides. As one of the prominent avian antimicrobial peptides, the expression of LEAP-2 was included in the experimental design. Results show that dietary GOS also influenced the HS-induced decrease in LEAP-2 expression. Taken together, it can therefore be recommended to further investigate the application of GOS as effective nutritional strategy to maintain the intestinal homeostasis under (heat) stress conditions.

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Supporting Information

Table S1. Feed composition standard broiler diet

Ingredients	Value (g/kg)	Nutrient	Value (g/kg)	Amino Acids	Value (g/kg)
Maize	322,20	Dry matter	879,60	Isoleucine	8,30
Wheat + xylanase	250,00	Crude ash	50,10	Leucine	15,93
Soya	225,00	Crude protein	204,20	Lysine	11,75
Rapeseed meal	30,00	Crude fat	70,80	Methionine	5,19
Peas (dry)	50,00	Crude fibre	30,50	Cysteine	3,44
Soy bean (heated)	50,00	Carbohydrates	525,30	Phenylalanine	9,67
Lard	20,00	Organic matter	146,00	Tyrosine	6,95
Soy bean oil	20,00	Starch	389,20	Threonine	7,96
Premix (maize)	5,00	Sugars	40,40	Tryptophan	2,33
Chalk	13,00	Neutral detergent fibre	99,30	Valine	9,34
Limestone	0,00	Acid detergent fibre	39,70	Arginine	13,14
Monocalcium phosphate	6,00	Calcium	7,90	Histidine	5,29
Salt (NaCl)	2,20	Total phosphorus	4,90	Alanine	9,33
$NaHCO_3$	2,00	Magnesium	1,60	Aspartic acid	19,42
Phytase	0,10	Potassium	9,00	Glutamic acid	38,27
L-lysine HCL	1,70	Sodium	1,48	Glycine	8,39
DL-methionine	2,20	Chlorine	2,00	Proline	12,33
L-threonine	0,60	Base-excess (mEq/kg)	235,50	Serine	9,85
		Net energy (MJ/kg)	10,40		
		Linoleic acid (MJ/kg)	26,80		
		Fe (mg/kg)	136,00		
		Mn (mg/kg)	21,00		
		Zn (mg/kg)	30,00		
		Cu (mg/kg)	6,00		

Chapter 6

Nitric oxide synthesis promoted by L-Arginine supplementation prevents intestinal epithelial cell injury under heat stress conditions: model experiments in Caco-2 cells

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Abstract

Heat stress (HS) is known to induce deleterious effects on the intestinal integrity by disrupting the intestinal epithelial junctional complexes. In this study we aimed to characterize the protective effect of L-Arginine (L-Arg) and its biosynthesized product, nitric oxide (NO) against HS-induced intestinal epithelial injury using an *in vitro* human epithelial colorectal adenocarcinoma (Caco-2) cell model. Caco-2 cells were grown on transwell inserts and pre-treated with different L-Arg concentrations (0.4, 1, 4 mM) followed by exposure to HS. Barrier integrity was determined by measuring Trans Epithelial Electrical Resistance (TEER) and epithelial permeability to Lucifer Yellow (LY). mRNA levels of the stress-related markers heat shock protein 70 (HSP70) and haem oxygenase-1 (HO-1) were analysed by qRT-PCR and NO production was measured. Additional sets of experiments were conducted using the inducible nitric oxide synthase (iNOS) inhibitor, L-NAME, to prevent the L-Arg-induced NO production and the subsequent changes in E-cadherin protein expression (western blot analysis) and localization (immunofluorescence staining) were assessed. L-Arg deprivation markedly increases the mRNA expression of HSP70 and HO-1 under HS conditions. Pre-treatment with 4 mM L-Arg could prevent the loss of intestinal epithelial integrity and mitigated the adverse effect of HS on E-cadherin expression and cellular distribution. This effect could be attributed to the stabilization of the cellular NO levels, as the inhibition of iNOS abrogated the effects of L-Arg. In turn, a significant drop in TEER values and an increase in LY permeability, as well as downregulation and delocalization of E-cadherin in HS-exposed cells could be observed, which could be mitigated by L-Arg supplementation. L-Arg supplementation protects the intestinal epithelial integrity by maintaining NO synthesis and stabilizing E-cadherin expression under HS conditions. The close correlation between a stabilized NO availability by L-Arg supplementation and the expression of E-cadherin was demonstrated for the first time in this study.

Introduction

L-Arginine (L-Arg), classified as a semi-essential amino acid, is involved in different physiological functions. The endogenous synthesis of L-Arg depends on cell type, age, developmental stage, diet, injuries and disease status [1,2]. Previous reviews have addressed the clinical potential of L-Arg supplementation, which has been shown to improve reproductive, cardiovascular, pulmonary, renal, gastrointestinal, liver and immune functions. Its therapeutic applications also includes patients with obesity, diabetes, and metabolic syndromes [2–4]. L-Arg serves as a precursor of physiologically important molecules, such as polyamines, creatine, agmatine, and most importantly nitric oxide (NO) [1]. The synthesis of NO is mediated by one of the constitutively expressed isoforms of nitric oxide synthase (NOS), such as neuronal NOS, and endothelial NOS as well as its inducible form iNOS, which is expressed in different cell types [5]. In the gastrointestinal tract, basal iNOS activity and NO production is required for tolerance to stress conditions, whereas excessive NO levels are destructive by enhancing epithelial cell apoptosis [6]. Tanaka *et al.* demonstrated that the protective effect of NO in the intestine is associated with increased mucus and fluid secretions as well as an inhibition of intestinal hypermotility [7]. Moreover, L-Arg-induced NO production has been introduced as a measure to mitigate inflammatory responses and to improve the intestinal integrity in experimental models of inflammatory bowel disease and hypoxia [8,9]. In mice, it is shown that after intestinal obstruction the L-Arg-induced NO production can also reduce the bacterial translocation by increasing the levels of the anti-inflammatory cytokine IL-10 and secretory immunoglobulin A (sIgA) in mice [10].

Exposure to high ambient temperatures or strenuous exercise or their combination can lead to heat stress (HS), which can progress into heat stroke, which is considered as a life threatening condition affecting the physiological function of different organs, including the intestines [11,12]. Various *in vitro* and *in vivo* investigations have described that HS can disrupt the intestinal integrity [13–16]. We and others previously could show that this HS-induced intestinal barrier dysfunction is associated with alterations in the expression and cellular location of tight junction (TJ) and adherens junction (AJ) proteins. A functional junctional complex is needed to seal the paracellular space between adjacent cells thereby preventing the penetration of luminal antigens and pathogens [14,17,18]. Especially, the E-cadherin expression and localization in the Caco-2 monolayer is targeted by HS, as published by our group before [14].

Previously, Costa *et al.* demonstrated that dietary supplementation with L-Arg is an effective intervention strategy to maintain the intestinal barrier integrity in mice forced to physical exercise under environmental HS conditions [19]. However, there is limited information about the various mechanisms by which L-Arg preserves the gastrointestinal barrier function. Therefore, in this study we investigated the effects of L-Arg supplementation and the associated NO production on the regulation

of (heat) stress responses and to linked these effects to intestinal barrier integrity, with a particular focus on E-cadherin, using a well-established *in vitro* epithelial colorectal adenocarcinoma (Caco-2) cell culture model.

Materials and Methods

Cell culture

Caco-2 cells were obtained from the American Type Tissue Collection (Code HTB-37) (Manassas, VA, USA, passages 5-19) and were cultured as described previously [14]. For all experiments, Caco-2 cells were seeded on 0.3 cm^2 high pore density polyethylene terephthalate membrane transwell inserts with 0.4 µm pores (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) placed in a 24-well plates (0.3×10^5 cells/transwell insert).

L-Arg and/or NG-Nitro-L-arginine (L-NAME) pre-treatment and HS exposure

After obtaining differentiated confluent Caco-2 monolayers at day 17-19 of culturing [Trans Epithelial Electrical Resistance (TEER) values in the range of 400 $\Omega.cm^2$], culture medium was replaced by L-Arg free DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with antibiotics (Penicillin 100 U/ml and Streptomycin 100 µg/ml) (Biocambrex, Verviers, Belgium) and different non-cytotoxic concentrations of L-Arg [0 mM (L-Arg deprivation), 0.4 mM (standard medium concentration), 1 mM or 4 mM] (Sigma-Aldrich, St. Louis, MO, USA). After 24h pre-incubation with different concentrations L-Arg, Caco-2 cells were subjected to control (37°C) or HS (42°C) conditions in a humidified atmosphere of 95% air and 5% CO_2 for 24h without changing the medium.

For iNOS inhibition studies, 12 mM L-NAME (Sigma-Aldrich) was added to Caco-2 monolayers (apical and basolateral) 1h prior to incubation with 4 mM L-Arg for 24h (in total 25h L-NAME incubation). This L-NAME concentration was based on previous data showing that three times more L-NAME than L-Arg is required for a competitive inhibition of iNOS [9].

TEER measurement

The integrity of the intestinal epithelial monolayer was assessed by measuring TEER using a Millicell-ERS voltohmmeter (Millipore, Temecula, CA, USA). Average TEER values of established Caco-2 cell monolayers prior to the start of the experiment were in the range of 400 ± 30 $\Omega.cm^2$. TEER was measured prior to and 24h after HS exposure, and results are expressed as a percentage of the initial value in an individual experiment.

Paracellular tracer flux assay

The paracellular permeability was investigated by measuring the transfer of Lucifer Yellow (LY, 0.457 kDa, 20 μg/ml, Sigma-Aldrich) across an established Caco-2 monolayer. 4h prior to the end of 24h HS challenge, LY was added to the apical compartment of the transwell inserts. At the end of HS challenge, the medium from the basolateral compartment was collected and the fluorescence intensity of LY was measured by a fluorometer (FLUOstar OPTIMA, Offenburg, Germany) at excitation and emission wavelengths of 410 nm and 520 nm, respectively.

RNA isolation and quantitative Real-Time PCR (qRT-PCR)

At the end of the experiment Caco-2 cells were collected for RNA extraction, cDNA synthesis and qRT-PCR analysis, according to previously described protocols [14], to assess the mRNA expression of Arginase II, HSP70 and HO-1. Table S1 represents the commercially manufactured sets of gene-specific primers (Eurogentec, Seraing, Belgium) and the corresponding annealing temperatures, which were used after confirmation of specificity and efficiency analysis by qRT-PCR with dilution series of pooled cDNA at a temperature gradient (55°C to 65°C) for primer annealing and subsequent melting curve analysis. The mRNA quantity of the target genes was calculated relative to the expression of β-Actin reference gene.

NO measurement

Since in a aqueous environment, like cell culture medium, NO is rapidly converted into the stable end products, nitrite and ultimately to nitrate, nitrite was measured by the Griess reaction as reported previously [20]. Briefly, 100 μl of culture medium from the apical compartment of transwell inserts were mixed with an equal volume of Griess reaction mix composed of 1% sulphanilamide (Sigma-Aldrich), and 0.1% N-(1-naphthyl)ethylenediamine (Sigma-Aldrich), in 5% H_3PO_4. The mixture was incubated for 5 min at room temperature and absorbance was assessed at 540 nm (FLUOstar OPTIMA, Offenburg, Germany) and compared with a sodium nitrite standard curve.

Western blot analysis

Caco-2 cells were lysed using 50 μl RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany). Total protein concentrations were measured using a BCA protein assay kit (Thermo Scientific). After the protein content of the samples was determined, lysates were normalized for protein content and western blot analysis was conducted as described previously [13], using antibodies against iNOS (1:1000, Thermo Fisher Sientific, Walthman, MA, USA) or E-cadherin (1:1000, eBioscience, San Diego, CA, USA), and β-actin (1:4000; Cell Signaling, Danvers, MA, USA) for equality of sample loading. Membranes were incubated with ECL Prime western blotting detection reagent (Amersham Biosciences, Roosendaal, The Netherlands) prior to obtaining

the digital images with the ChemiDoc™ MP imager (Bio-Rad, Hercules, CA, USA). The ImageJ 1.47 software was used to quantify the signal intensity of western blot bands. The protein levels were normalized with β-Actin and are expressed as mean fold change in comparison with the control group.

Immunofluorescence staining

The inserts with Caco-2 cells were fixed with 10% formalin for 10 min. After washing with PBS, the immunofluorescence staining was performed to determine the cellular localization of iNOS and E-cadherin as described previously [14], using the primary antibodies against iNOS (1:100, Thermo Fisher Sientific, Walthman, Massachusetts, USA) and E-cadherin (1:50, BD Biosciences, San Diego, CA, USA) followed by incubation with Alexa-Fluor conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). After performing a nuclear counterstaining with Hoechst 33342 (1:2000, Invitrogen), the inserts were mounted with FluorSave™ Reagent (Calbiochem, Schwalbach, Germany) and immune-localization of iNOS and E-cadherin was determined using a Nikon Eclipse TE2000-U microscope equipped with a Nikon Digital Sight DS-U1 camera (400×).

Statistical analysis

Results are expressed as means ± SEM of 3 independent experiments (n=3), each performed in triplicate. Analyses were performed by using GraphPad Prism (version 6.05) (GraphPad, La Jolla, CA, USA). Differences between groups were statistically determined by using Two-way analysis of variance (ANOVA), with Bonferroni post-hoc test. Results are considered statistically significant when $P < 0.05$.

Results

L-Arg deprivation modulates the mRNA expression of HSP70 and HO-1 under HS conditions

Exposure to HS for 24h resulted in an upregulation in the HSP70 (Fig. 1A) and HO-1 (Fig. 1B) mRNA expression in Caco-2 cells. This increase was more pronounced in the absence of L-Arg (L-Arg free medium) compared to 0.4 mM L-Arg (standard medium concentration). The L-Arg deprivation resulted in an approximately 120-fold increase in HSP70 mRNA expression and an about 20-fold increase in HO-1 mRNA expression under HS conditions. Although HS exposure induced a drop in TEER values and increased the LY permeability across the intestinal monolayer, the effects of L-Arg deprivation on these two markers for intestinal integrity were not significantly different from the corresponding values obtained in cells exposed to the complete medium containing 0.4 mM L-Arg (Fig. 1C and 1D).

L-Arg increases the HSP70 mRNA expression concentration-dependently and partly prevents the disruption of intestinal integrity under HS conditions

Pre-incubation of a Caco-2 cell monolayer prior to HS with different concentrations of L-Arg showed a concentration-dependent effect, as only the highest concentration of 4 mM L-Arg led to a significantly increased mRNA expression of HSP70 in HS-exposed Caco-2 cells, whereas in the HS-exposed cells incubated with 0.4 mM (standard medium concentration) and 1 mM L-Arg no significant differences were observed (Fig. 2A). Moreover, no significant differences were found between the effects of the different L-Arg concentrations on the HS-induced HO-1 mRNA expression (Fig. 2B). Pre-treatment of Caco-2 cells with 4 mM L-Arg restored the HS-induced drop in TEER values significantly, compared to the 0.4 and 1 mM L-Arg (Fig. 2C). Although HS exposure increased the LY permeability and 4 mM L-Arg prevented this increase, these effects were not significantly different from the corresponding reference group (Fig. 2D).

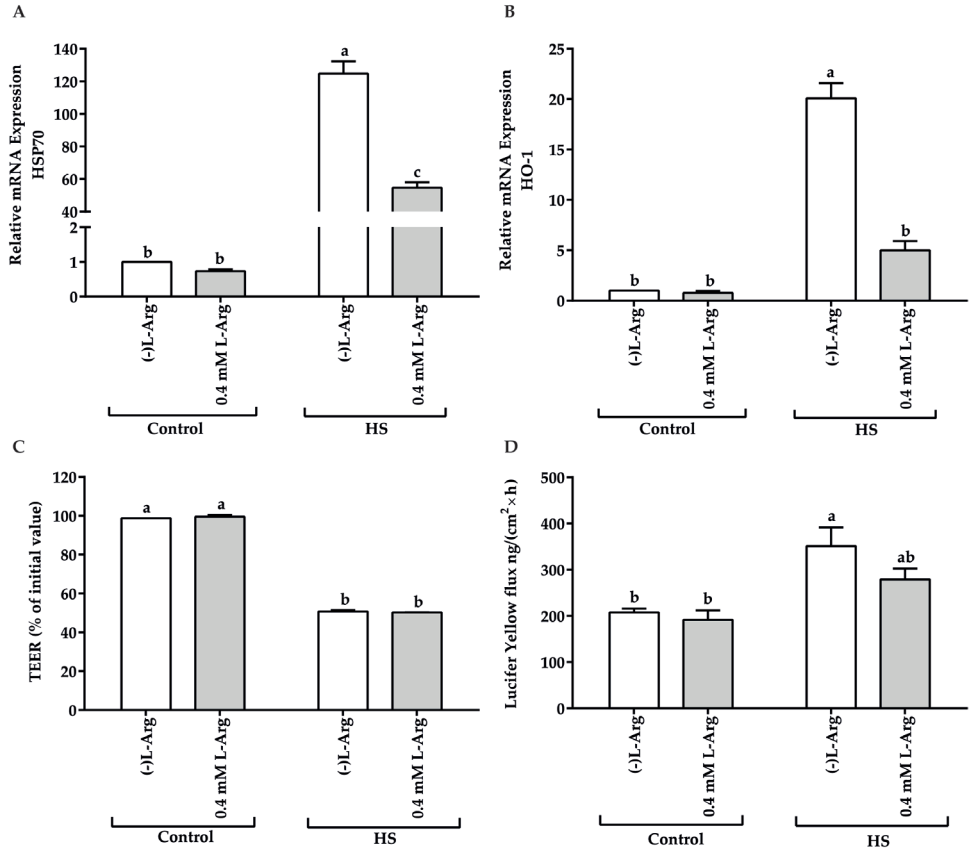

Figure 1. L-Arg deprivation markedly increases the mRNA expression of HSP70 and HO-1 under HS conditions. Caco-2 cells grown on inserts were pre-treated with L-Arg free medium [(-)L-Arg] or 0.4 mM L-Arg (standard medium concentration) for 24h prior to exposure to control (37°C) or HS conditions (42°C) for 24h. HSP70 (A) and HO-1 (B) mRNA expression was assessed by qRT-PCR and TEER levels (C) as well as LY transport (D) across the Caco-2 monolayer were measured. Results are expressed as relative mRNA expression normalized to β-actin (A,B), percentage of initial TEER value (C), the amount of tracer transported [ng/(cm^2 × h)] (D), as means ± SEM of three independent experiments each performed in triplicate. Different lower-case letters denote significant differences among groups. L-Arg increases the HSP70 mRNA expression concentration-dependently and partly prevents the disruption of intestinal integrity under HS conditions.

L-Arg supplementation prevents the HS-induced decrease in NO production

Measurement of the NO production (by means of nitrite) revealed that exposure to HS significantly decreased cellular NO levels. A comparison between L-Arg deprived Caco-2 cells and cells exposed to a standard medium concentration of L-Arg (0.4 mM) showed, however, no significant differences (Fig. 3A). Supplementation of L-Arg increased the measurable nitrite concentration under control and HS conditions in a concentration-dependent manner. This increase was only significant

under HS conditions at a concentration of 4 mM L-Arg and an almost complete restoration of NO levels was achieved (Fig. 3B).

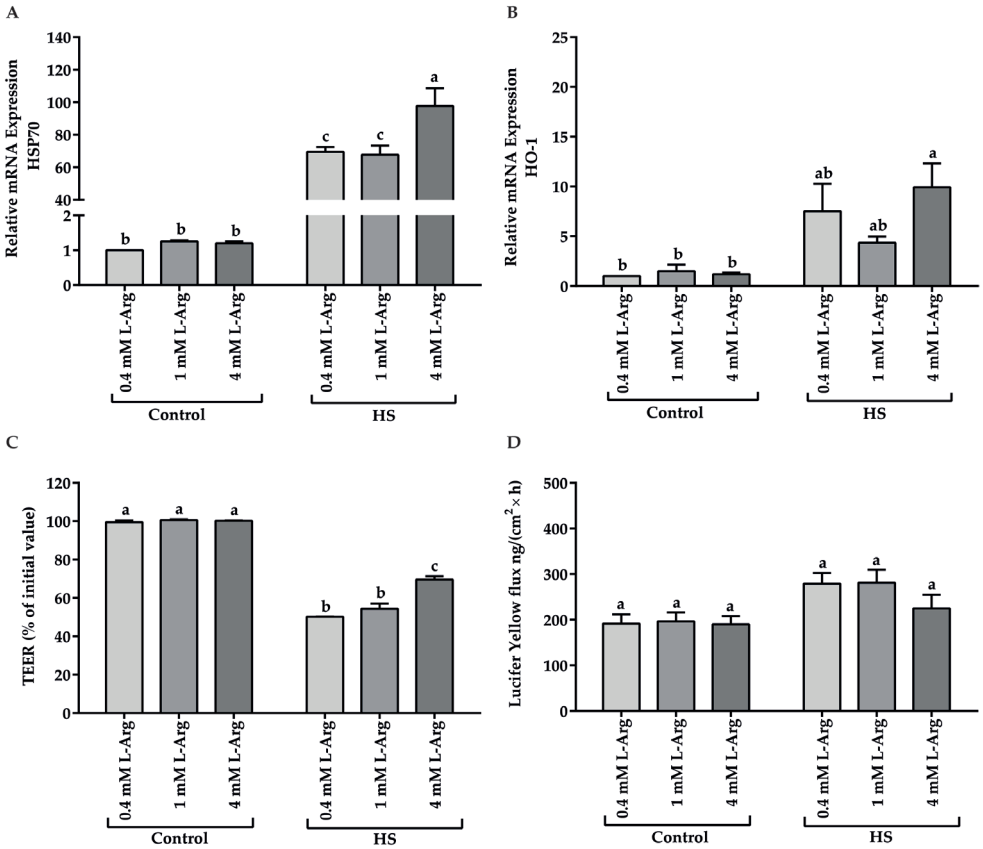

Figure 2. Supplementation with L-Arg increases the HSP70 mRNA expression and partly prevents the disruption of intestinal integrity under HS conditions. Caco-2 cells grown on inserts were pre-treated with different concentrations L-Arg (0.4, 1, 4 mM) for 24h prior to exposure to control (37°C) or HS conditions (42°C) for 24h. HSP70 (A) and HO-1 (B) mRNA expression was assessed by qRT-PCR and TEER levels (C) as well as LY transport (D) across the Caco-2 monolayer were measured. Results are expressed as relative mRNA expression normalized to β-Actin (A,B), percentage of initial TEER value (C), the amount of tracer transported [ng/(cm^2 × h)] (D), as means ± SEM of three independent experiments each performed in triplicate. Different lower-case letters denote significant differences among groups.

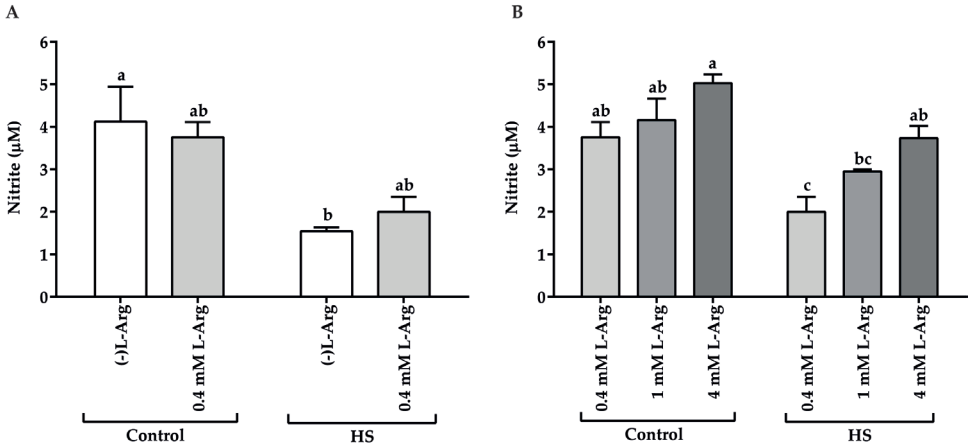

Figure 3. Supplementation with L-Arg prevents the HS-induced decrease in NO production. Caco-2 cells grown on inserts were pre-treated either with L-Arg free medium [(-)L-Arg] and 0.4 mM L-Arg (standard medium concentration) (A) or with different concentrations L-Arg (0.4, 1, 4 mM) (B) for 24h prior to exposure to control (37°C) or HS conditions (42°C) for 24h. Production of nitrite, a stable metabolite of NO, was measured by a Griess reaction. Results are expressed as means ± SEM of three independent experiments, each performed in triplicate. Different lower-case letters denote significant differences among groups.

L-Arg prevents the HS-induced decrease in iNOS protein levels

Western blot analysis showed that HS exposure significantly reduced the iNOS protein levels in cells cultured in L-Arg-free medium. Pre-treatment with 4 mM L-Arg prevented this HS-induced decrease of cellular iNOS levels (Fig. 4A). In agreement with the western blot results, visualization of the cellular localization of iNOS by immunofluorescence staining demonstrated that HS exposure under L-Arg deprivation reduced the number of clearly iNOS-positive cells and this decrease in iNOS-positive cells could be prevented by pre-treatment with 4 mM L-Arg (Fig. 4B).

L-NAME reverses the protective effect of L-Arg

To confirm that the effects of supplementary L-Arg were directly related to the iNOS-dependent induction of cellular NO levels in all experiments, additional incubations were performed in the presence of L-NAME, the prototypic inhibitor of iNOS.

Inhibition of iNOS by L-NAME completely abolished the protective effect of L-Arg, even at the highest concentration of 4 mM L-Arg under HS conditions as demonstrated by western blot analysis and immunofluorescence staining for iNOS (Fig. 4A and 4B) as well as NO production (Fig. 5). Additionally, L-NAME also reduced the measurable iNOS protein levels under thermal neutral conditions with or without L-Arg supplementation (Fig. 4A).

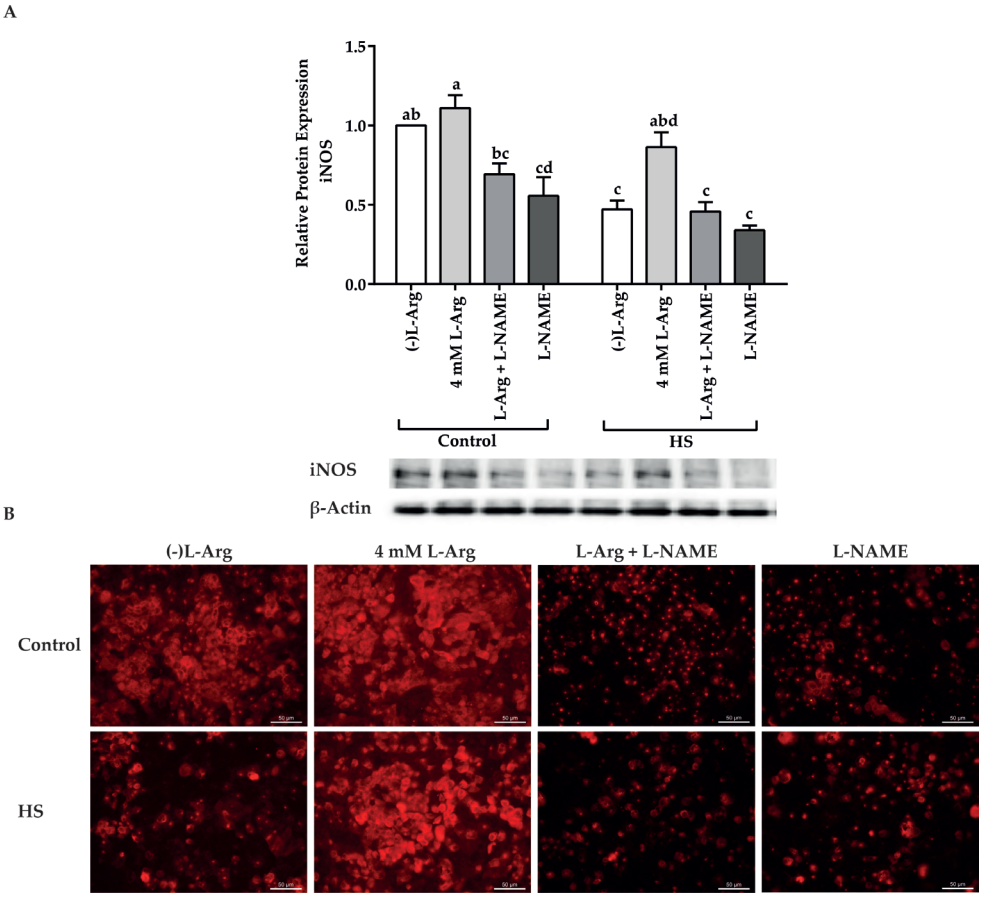

Figure 4. L-Arg prevents the HS-induced decrease in iNOS protein levels. Caco-2 cells grown on inserts were pre-treated with L-Arg free medium [(-)L-Arg] or 4 mM L-Arg in presence or absence of L-NAME (12 mM) prior to exposure to control (37°C) or HS conditions (42°C) for 24h. Protein expression (A) and cellular localization (B) of iNOS was determined by western blot analysis and immunofluorescence staining, respectively. Western blot results are expressed as relative protein expression, normalized to β-Actin, as means ± SEM of three independent experiments. Different lower-case letters denote significant differences among groups. Representative immunofluorescence pictures (B) of the Caco-2 cells are stained with an antibody against iNOS (400× magnification). Scale bars represent 50 μm.

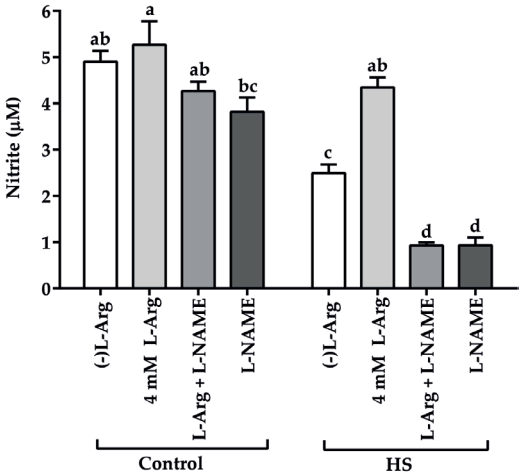

Figure 5. L-NAME reverses the protective effect of L-Arg. Caco-2 cells grown on inserts were pre-treated with L-Arg free medium [(-)L-Arg] or 4 mM L-Arg in presence or absence of L-NAME (12 mM) prior to exposure to control (37°C) or HS conditions (42°C) for 24h. Production of nitrite, a stable metabolite of NO, was measured by a Griess reaction. Results are expressed as means ± SEM of three independent experiments, each performed in triplicate. Different lower-case letters denote significant differences among groups.

L-Arg induced iNOS production prevents the HS-related disruption of intestinal integrity

As shown in Fig. 6A, pre-treatment with 4 mM L-Arg prior to HS exposure to Caco-2 cells significantly prevented the HS-induced TEER decrease under L-Arg deprivation and this effect of L-Arg was abolished after inhibition of iNOS by L-NAME (Fig. 6A). In addition, inhibition of iNOS exacerbated the HS-induced TEER drop in Caco-2 cells exposed to L-Arg free medium.

The HS-induced TEER drop was accompanied with an increased transfer of LY across the intestinal epithelial monolayer. Subsequently, L-Arg pre-treatment significantly prevented the increased LY flux under HS conditions, whereas inhibition of iNOS eliminated this protective effect of L-Arg (Fig. 6B). L-NAME pre-treatment also aggravated the HS-induced increase in paracellular (LY) permeability in Caco-2 exposed to L-Arg free medium.

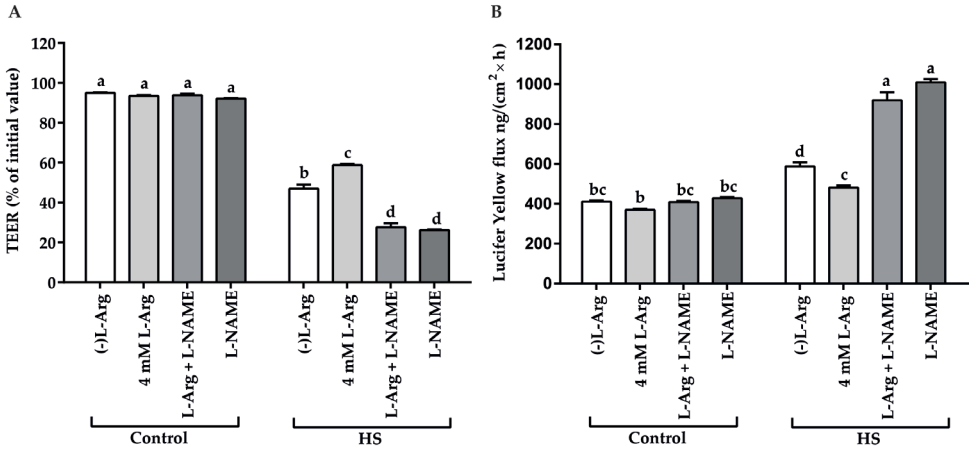

Figure 6. L-Arg induced iNOS production prevents the HS-induced disruption of intestinal integrity. Caco-2 cells grown on inserts were pre-treated with L-Arg free medium [(-)L-Arg] or 4 mM L-Arg in presence or absence of L-NAME (12 mM) prior to exposure to control (37°C) or HS conditions (42°C) for 24h to measure the TEER levels (A) or LY (B) transport across the Caco-2 monolayer. Results are expressed as percentage of initial TEER value (A), or the amount of tracer transport [ng/(cm^2 × h)] (B) as means ± SEM of three independent experiments each performed in triplicate. Different lower-case letters denote significant differences among groups.

6

L-Arg promotes the expression of E-cadherin and prevents the HS-induced downregulation and delocalization of E-cadherin

A comparison of results from western blot analyses performed with L-Arg deprived cells and cells supplemented with 4 mM L-Arg indicated that L-Arg promotes the expression of E-cadherin under control conditions. This positive effect was abolished by L-NAME. Under HS conditions, L-Arg pre-treatment could significantly prevent the HS-induced downregulation of E-cadherin protein expression and again L-NAME abolished this L-Arg-induced effect (Fig. 7A).

Immunofluorescence staining revealed that HS-exposed cells under L-Arg deprivation exhibited irregular structures, suggesting clumping and internalization of fragmented E-cadherin. Pre-treatment with L-Arg retained the membrane-associated localization of E-cadherin, whereas L-NAME abolished this preventive effect of L-Arg. Representative examples of the differences between L-Arg-deprived cells and cells supplemented with 4 mM L-Arg with or without inhibition of iNOS under control and HS conditions are depicted in Fig. 7B.

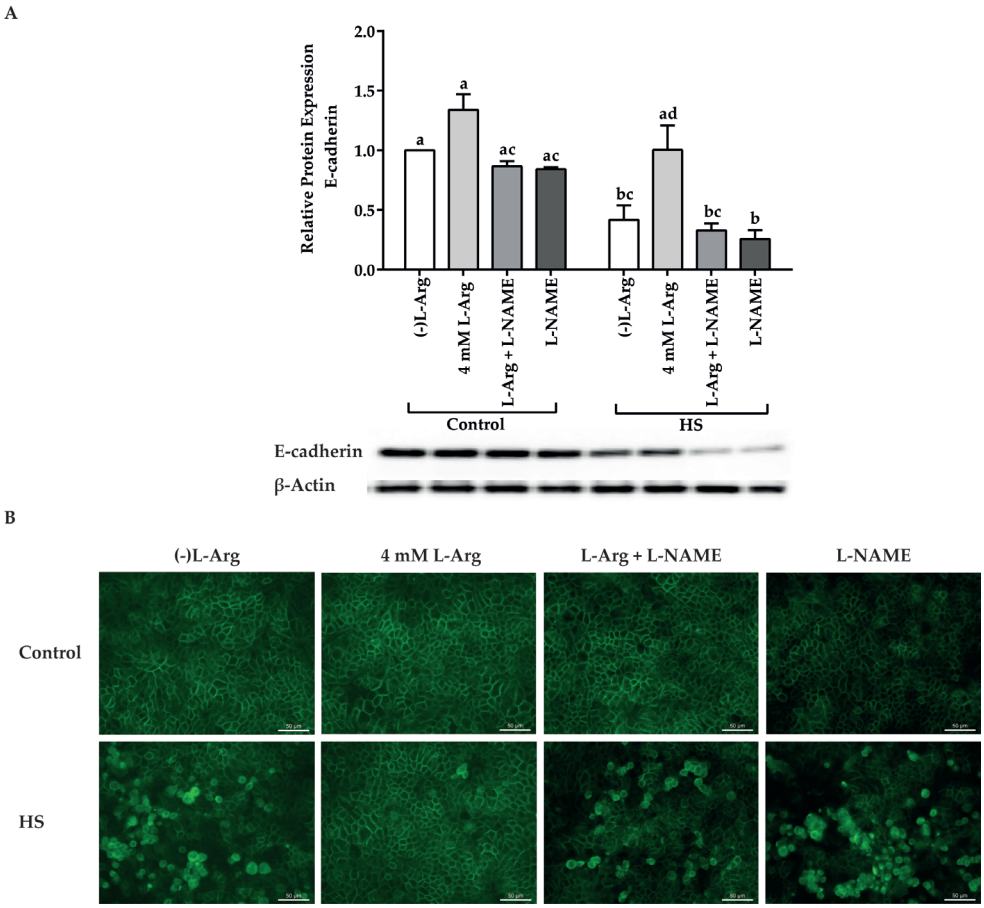

Figure 7. L-Arg induced iNOS production prevents the HS-induced downregulation and delocalization of E-cadherin. Caco-2 cells grown on inserts were pre-treated with L-Arg free medium [(-)L-Arg] or 4 mM L-Arg in presence or absence of L-NAME (12 mM) prior to exposure to control (37°C) or HS conditions (42°C). Protein expression (A) and cellular localization (B) of E-cadherin was determined by western blot analysis and immunofluorescence staining, respectively. Western blot results are expressed as relative protein expression, normalized to β-Actin, as means ± SEM of three independent experiments. Different lower-case letters denote significant differences among groups. Representative immunofluorescence pictures (B) of the Caco-2 cells are stained with an antibody against E-cadherin (400× magnification). Scale bars represent 50 μm.

Discussion

The *de novo* synthesis of arginine in cells occurs mainly from citrulline and aspartic acid forming arginine-succinate, which is further converted into L-Arg and fumaric acid, a reaction catalysed by the enzyme argininosuccinate lyase [21]. In the urea cycle, L-Arg can be converted back to L-citrulline and then be recycled back to L-Arg if required [21]. The capacity of cells to synthesize L-Arg from ornithine, citrulline and argininosuccinate determines their vulnerability to arginine depletion, which can occur under conditions of a reduced blood flow and hypoxia, as well as other stress conditions. Moreover, cells that need to proliferate have an excessive need for L-Arg. This explains among others the dependency of intestinal epithelial cells on sufficient L-Arg supplies [22].

Previously, we and others showed that HS affects gut barrier function and increases the intestinal epithelial permeability [13–15]. Considering these deleterious effects of HS on the intestinal epithelium [23], we aimed to investigate more specifically the effect of L-Arg on epithelial stress injury using the human intestinal epithelial Caco-2 cells as a model.

In this model, we first investigated the typical markers of HS and the associated oxidative stress response, by measuring the mRNA expression levels of HSP70 and HO-1. In a standard medium, which contains 0.4 mM L-Arg, an increase in the expression of HSP70 and HO-1 was observed after rising the incubation temperature to 42°C for 24h to induce a heat shock response (HSR). Under the conditions of L-Arg shortage (medium without any L-Arg) this upregulation of HSP70 and HO-1 was even more pronounced, suggesting that the endogenous cellular synthesis of L-Arg is not sufficient to meet the L-Arg needs under HS conditions. Surprisingly, the highest tested concentration of 4 mM L-Arg stimulated also the HSP70 mRNA expression compared to the lower L-Arg concentrations (1 and 0.4 mM) under HS conditions. As L-Arg is the precursor of NO in its endogenous synthesis catalysed by iNOS, these apparently controversial responses are likely to reflect the divergence of NO effects: at moderate cellular concentrations, NO acts as radical scavenger, whereas high concentrations result in the formation of nitrogen radicals, promoting cellular oxidative stress [5,24–26].

To further confirm that the protective effects of L-Arg supplementation on intestinal integrity are directly related to the cellular NO levels, we inhibited iNOS by pre-treating the cells with the prototypic inhibitor, L-NAME. Inhibition of iNOS completely abolished the protective effect of L-Arg supplementation on the intestinal barrier dysfunction under HS conditions, as measured by a decrease in TEER levels and a corresponding higher LY flux across the intestinal epithelial monolayer.

In the Caco-2 model used in the current study, the TJ proteins were not affected by HS, as previously reported by our group [14]. However, a clear effect of HS on AJ E-cadherin mRNA and protein expression and cellular distribution could

be demonstrated, an effect that could be prevented by L-Arg supplementation as mentioned above. Again, NO appears to be responsible for the regulation of E-cadherin expression, since inhibition of iNOS eliminated the protective effect of L-Arg on E-cadherin. These findings are in line with the outcome of the study from Vyas-Read *et al.* who showed that L-Arg-induced NO prevents the downregulation of E-cadherin in lung epithelial cells stimulated with ransforming growth Factor-β (TGF-β1) [27]. Moreover, Nagarajan *et al.* demonstrated that supplementation with exogenous NO protects and recovers endothelial membrane integrity by increasing the endothelial cGMP content and by rearrangement of actin polymerization possibly leading to regulation of AJs, including cadherin and β-catenin [28].

The L-Arg-induced increase in cellular NO protects cells also from lipid peroxidation, another typical sign of HS [29]. As NO is known to scavenge free radicals as mentioned above, the protective effect of L-Arg on barrier function and expression of AJ proteins, may be also attributable to the decrease in lipid peroxidation [9].

This hypothesis is supported by the results of the immunofluorescent staining, as we showed that L-Arg supplementation preserved the adherence of E-cadherin to the (lipid-rich) cell membrane under HS conditions, while L-Arg deprivation or inhibition of iNOS resulted in an irregular distribution of E-cadherin in these HS-exposed cells.

A less commonly addressed effect of NO is its capability to attenuate the HS-induced protein tyrosine phosphorylation [30]. Tyrosine phosphorylation under HS conditions is thought to be involved in the dissociation of occludin-ZO1 TJs and the E-cadherin-β-catenin AJs complexes [30,31], resulting in the functional impairment of the TJ complex and loss of barrier integrity. Inhibition of tyrosine phosphorylation can therefore also contribute to the protective effects of L-Arg induced NO.

Finally, entirely different pathways involved in the L-Arg metabolism may also contribute to its protective effect. The Arginase pathway yields also polyamines, which can regulate the expression of E-cadherin and thus play a direct role in the maintenance of intestinal epithelial integrity [32]. Although this study did not focus on the Arginase pathway, HS-exposed Caco-2 cells in Arg-deficient medium showed a significant higher Arginase II mRNA expression compared to standard medium conditions (0.4 mM L-Arg) (Fig. S1). It can be suggested that this increase can be induced by the HS-induced oxidative stress response, since it is described that hypoxia increases the Arginase activity as well as Arginase II mRNA and protein expression via protein kinase C/RhoA/Rho kinase (ROCK), mitogen-activated protein kinase, tyrosine kinases and cyclic adenosine monophosphate/protein kinase A pathways [33]. Pre-treatment with 4 mM L-Arg significantly increased the Arginase II mRNA expression compared to the lower L-Arg concentrations (Fig. S1), suggesting that the polyamine production is also important for the L-Arg-induced improvement of epithelial barrier integrity.

The protective effect of L-Arg supplementation on intestinal integrity under stress

conditions has been demonstrated in different animal models [8,9,19,31,34,35]. *In vivo*, a loss of barrier integrity results in the translocation of intestinal antigens and pathogens and a pronounced inflammatory response [17]. HSP70 has an inhibitory effect on the I-κB/NF-κB pathway, and subsequently on the iNOS gene expression, thereby tempering an excessive NO synthesis [36–39]. It is also known that inducible HSP70 couples to iNOS and its critical transcription factor (Kruppel-like factor 6), which in turn leads to an inhibition of iNOS gene expression [40]. Some *in vivo* studies have shown, however, that exposure to HS increases the production of NO under conditions of endotoxemia following HS and this seems to represent a late inflammatory response [41,42].

Conclusions

Our results indicate that L-Arg can protect the intestinal epithelial integrity by preventing the HS-induced reduction in iNOS-mediated NO synthesis. The close correlation between a stabilized NO availability via L-Arg supplementation and the expression of E-cadherin, as part of the AJ complex responsible for the integrity of the epithelial barrier, was demonstrated for the first time in this study.

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Supporting Information

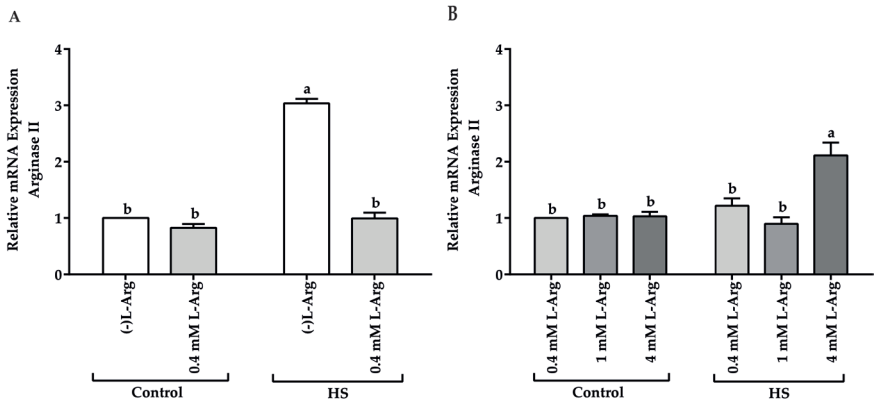

Figure S1. L-Arg depletion and 4 mM L-Arg increase Arginase II mRNA expression. Caco-2 cells grown on inserts were pre-treated either with L-Arg free medium [(-)L-Arg] and 0.4 mM L-Arg (standard medium concentration) (A) or with different concentrations L-Arg (0.4, 1, 4 mM) (B) for 24h prior to exposure to control (37°C) or HS conditions (42°C) for 24h. Arginase II mRNA expression was assessed by qRT-PCR. Results are expressed as relative mRNA expression, normalized to β-actin, as mean ± SEM of three independent experiments, each performed in triplicate. Different lower-case letters denote significant differences among groups.

Table S1. Primer sequences used for qRT-PCR

	Primer sequence (5′-3′)			
Genes	**Forward**	**Reverse**	**AT**	**References**
β-Actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	63	NM_001101
Arginase II	GAAGAAATCCGTCCACTCCG	GGACCATGCTCCACTCCTTTT	59	[43]
HSP70	AGAGCCGAGCCGACAGAG	CACCTTGCCGTGTTGGAA	57	NG_011855.1
HO-1	GCCACCAAGTTCAAGCAGCT	CAGTGCCCACGGTAAGGAAG	61.2	NM_002133.2

AT, Annealing temperature (°C)

Chapter 7

α-lipoic acid ameliorates the intestinal epithelial monolayer damage under heat stress conditions: model experiments in Caco-2 cells

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Abstract

Under conditions of high ambient temperatures and/or strenuous exercise, humans and animals experience considerable heat stress (HS) leading among others to intestinal epithelial damage through induction of cellular oxidative stress. The aim of this study was to characterize the effects of α-lipoic acid (ALA) on HS-induced intestinal epithelial injury using an *in vitro* colorectal adenocarcinoma (Caco-2) cell model. A confluent monolayer of Caco-2 cells were pre-incubated with ALA (24h) prior to control (37°C) or HS conditions (42°C) for 6h or 24h and the expression of heat shock protein 70 (HSP70), heat shock factor 1 (HSF1) and the antioxidant nuclear factor erythroid 2-related factor-2 (Nrf2) were investigated. Intestinal integrity was determined by measuring Trans Epithelial Electrical Resistance (TEER), paracellular permeability, junctional complex reassembly and E-cadherin expression and localization. Furthermore, cell proliferation was measured in an epithelial wound healing assay and the expression of the inflammatory markers cyclooxygenase-2 (COX-2) and transforming growth Factor-β (TGF-β) were evaluated. ALA pre-treatment increased the HSP70 mRNA and protein expression under HS conditions, but did not significantly modulate the HS-induced activation of HSF1. The HS-induced increase in Nrf2 gene expression as well as the Nrf2 nuclear translocation was hampered by ALA. Moreover, ALA prevented the HS-induced impairment of intestinal integrity. Cell proliferation under HS conditions was improved by ALA demonstrated in an epithelial wound healing assay and ALA was able to affect the HS-induced inflammatory response by decreasing the COX-2 and TGF-β mRNA expression. ALA can prevent the disruption of intestinal integrity, enhances epithelial cell proliferation, and reduces the inflammatory responses under HS conditions in an *in vitro* Caco-2 cell model.

Introduction

α-lipoic acid (ALA, 1, 2-dithiolane-3-pentanoic acid), is present in all kinds of pro- and eukaryote cells and is considered to be one of the most potent cellular antioxidants. ALA exhibits free radical scavenging properties, regulates antioxidant enzymes, has metal-chelating capacity, interacts with other antioxidants (vitamin C and E) [1,2] and maintains its antioxidant function in both oxidized (disulfide, oALA) and reduced (di-thiol; dihydro-lipoic acid, DHLA) forms [2]. In turn, ALA has been suggested as a treatment for different pathologies associated with redox imbalances, including diabetes, ischemia-reperfusion injury and heavy metal poisoning [3–5]. Besides the antioxidant activities, more recent investigations have demonstrated that ALA has anti-inflammatory properties and the therapeutic potential of ALA has been described in various inflammatory disorders [6,7]. Trivedi and Jena demonstrated the protective effects of ALA against gut hyper-permeability and the associated systemic inflammation in a murine model of ulcerative colitis [8]. In addition, *in vitro* and *in vivo* investigations by Fan *et al.* showed a protective effect of ALA on the intestinal barrier function, which can be related to its antioxidant effect and to the increase in the expression of tight junction (TJ) proteins [9].

Heat stress (HS), experienced by humans and animals under conditions of high ambient temperatures and/or strenuous exercise, is known to disturb the balance between the production of reactive oxygen species (ROS) and the antioxidant defence system at the cellular level, resulting in oxidative stress [10]. A hallmark in the protection of cells against HS, is the production of heat shock proteins (HSPs), acting as chaperones in the folding and refolding of cellular proteins [11]. HSPs genes are transcriptionally activated by heat shock factor 1 (HSF1) and the mechanism by which HSPs, in particular HSP70, maintains protein homeostasis during (heat) stress, is the inhibition of the protein aggregation and misfolding, thus preventing their irreversible denaturation [11].

We and others have previously shown that dysfunction of the intestinal barrier can be caused by HS and can lead to increased intestinal permeability and corresponding inflammatory responses [12–14]. Considering the beneficial effects of ALA as an antioxidant and its role in the protection of the intestinal barrier function, we hypothesized that ALA can prevent HS-induced cellular dysfunction and disruption of intestinal barrier integrity.

Hence, we determined the regulatory effects of ALA against HS-induced intestinal epithelial damage, using a well-established epithelial colorectal adenocarcinoma (Caco-2) cell culture model and monitored the response to HS, measuring redox regulation, intestinal integrity, cell proliferation (wound healing assay) and the corresponding inflammatory response. The obtained *in vitro* results contribute to the understanding of the mechanisms of action of ALA and suggest that ALA is a promising candidate to be used as a food additive to increase the resilience to HS.

Materials and Methods

Cell culture

Caco-2 cells, were obtained from the American Type Tissue Collection (Code HTB-37) (Manassas, VA, USA, passages 5-19) were cultured as described previously [12]. Cells were seeded on 0.3 cm^2 high pore density polyethylene terephthalate membrane transwell inserts with 0.4 μm pores (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) placed in a 24-well plate (0.3×10^5 cells/transwell insert) and the transwell experiments were conducted after obtaining a differentiated confluent Caco-2 monolayer at day 17-19 of culturing. For the wound healing and nuclear protein extraction assays, Caco-2 cells were seeded at a density of 1×10^5 cells/well in 6-well plates and experiments were started after 10 days and 17 days of culturing, respectively.

ALA pre-treatment and HS-exposure

ALA was obtained from Sigma-Aldrich [(±)-α-lipoic acid, St. Louis, MO, USA)]. Prior to HS-exposure, Caco-2 cells were pre-treated with DMEM (control) or DMEM supplemented with different clinically relevant concentrations of ALA (15 μM, 30 μM, 60 μM) [15,16] added to the apical and basolateral compartments for 24h [17]. Caco-2 cells were subjected to 37°C or HS conditions (42°C) in a humidified atmosphere of 95% air and 5% CO_2 for 6 or 24h. Neither HS-exposure for 24h, nor ALA pre-treatment in the used concentration affected the Caco-2 cell viability measured by lactate dehydrogenase release (Fig. S1).

RNA extraction and quantitative Real-Time PCR (qRT-PCR)

The mRNA expression of different target genes [HSP70, nuclear factor erythroid 2-related factor-2 (Nrf2), cyclooxygenase-2 (COX-2), transforming growth factor-β (TGF-β)] were measured by qRT-PCR in Caco-2 cells pre-treated with different concentrations of ALA for 24h followed by exposure to HS for 6h. RNA extraction, cDNA synthesis and qRT-PCR analysis were performed according to a previously described protocol [12]. Forward and Reverse primers (Eurogentec, Seraing, Belgium) with corresponding annealing temperatures are represented in Table S1. The mRNA quantity was calculated relative to the expression of β-Actin reference gene.

Western blot analysis

Caco-2 cells pre-treated with different ALA concentrations for 24h and exposed to HS for 6h (HSF1, Nrf2) or 24h (HSP70, E-cadherin) were lysed using 50 μl RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany). Lysates were normalized for protein content and western blot analysis was conducted as described previously [13] using primary antibodies against HSF1 (1:1000; Cell Signaling, Danvers, MA, USA),

HSP70 (1:1000; Enzo Life Sciences, Farmingdale, NY, USA) or E-cadherin (1:1000, eBioscience, San Diego, CA, USA), and β-Actin antibody (1:4000; Cell Signaling) for equality of sample loading. For the detection of Nrf2, the nuclear protein extracts were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents based on the manufacturer's instructions (Pierce, Rockford, IL, USA). Nuclear protein concentrations were measured, normalized and the western blot analysis was conducted using primary antibodies against Nrf2 (1:1000; Cell Signaling), and Lamin A (1:1000; Cell Signaling) for equality of loading. Digital images were obtained with ChemiDoc™ MP imager (Bio-Rad, Hercules, CA, USA) and signal intensities were quantified using the ImageJ 1.47 software.
The protein expression was normalized with β-Actin or Lamin A (for nuclear proteins) and expressed as mean fold change in relation to the control group.

Immunofluorescence staining
Cellular localization of HSF1 and E-cadherin was assessed by an immunofluorescence staining as described previously [12]. Briefly, after HS-exposure cells were fixed with 10% formalin, washed with PBS and permeabilized with PBS containing 0.1% Triton-X-100. After blocking in 5% serum, cells were incubated with anti-HSF1 (1:100, Cell Signaling) and anti-E-cadherin (1:50, eBioscience) antibodies for 2h at room temperature followed by incubation with Alexa-Fluor conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). After a nuclear counterstaining with Hoechst 33342 (1:2000; Invitrogen), the inserts were mounted with FluorSave™ Reagent (Calbiochem) and immune-localization of HSF1 and E-cadherin was determined using a Nikon Eclipse TE2000-U microscope equipped with a Nikon Digital Sight DS-U1 camera (400×). HSF1-immunostained slides were also examined by a Leica TCS SPE-II confocal laser scanning-microscope on a DMI4000 (Leica Microsystems, Wetzlar, Germany), images were acquired with an oil-immersion objective (63×) and assembled using Image J 1.47 software.

Trans Epithelial Electrical Resistance (TEER) measurement
The integrity of the transwell-grown Caco-2 monolayers was determined by measuring TEER using a Millicell-ERS voltohmeter (Millipore, Temecular, CA, USA). Average TEER values prior to the start of the experiment were in the range of $400 \pm 30\ \Omega.cm^2$. Results are expressed as a percentage of initial value.

Paracellular permeability assay
The paracellular permeability across the Caco-2 monolayer was investigated by measuring the Lucifer Yellow (LY, 0.457 kDa, 20 μg/ml; Sigma) flux. LY was added to the apical compartment (4h prior to the end of HS-exposure) for 4h and the fluorescence intensity of LY in the basolateral compartment was measured by fluorometer (FLUOstar OPTIMA, Offenburg, Germany) at excitation and emission wavelengths of 410 and 520 nm respectively.

Calcium switch assay

Caco-2 cells grown on inserts were pre-treated with different ALA concentrations added to the apical and basolateral compartment for 24h. Subsequently, cells were washed with PBS and cell-cell contacts were disrupted by incubation with 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, Sigma) in calcium- and magnesium-free HBSS (Gibco, Invitrogen, Carlsbad CA, USA) for 20 min. After the incubation, HBSS-EGTA was removed, cells were rinsed and cell-cell junctions were allowed to re-establish by incubation with either complete cell culture DMEM (containing 2 mM $CaCl_2$) or in DMEM supplemented with ALA concentrations. TEER values were measured at different time points (0, 2, 4, 6, 8h) during this recovery period [18].

Wound healing assay

Confluent Caco-2 monolayers seeded in 6-well plates (1×10^5 cells/well), were pre-treated with different concentrations of ALA for 24h. The wound healing assay was conducted as previously described [19]. Briefly, standardized wounds were created in Caco-2 monolayers by scratching with a 200 μl pipette tip (Greiner Bio-One, Frickenhausen, Germany) across the maximum diameter of each well. Thereafter, detached cells were removed by washing with PBS and the cells were incubated with DMEM or DMEM supplemented with ALA under control or HS conditions for 24h. Immediately after scratching as well as 24h after scratching, phase contrast images were acquired with the Olympus CKX41 microscope (Olympus Co. Ltd., Tokyo, Japan) and wound widths were measured using digital imaging system software (Leica Application Suite V4.2). Data were calculated as percentages of wound area relative to the initial wound width.

Statistical analysis

Results are expressed as means ± SEM of 3 independent experiments (n=3), each performed in triplicate. Analyses were performed by using GraphPad Prism (version 6.05) (GraphPad, La Jolla, CA, USA). Differences between groups were statistically determined by using Two-way analysis of variance (ANOVA), with Bonferroni post-hoc test. For the calcium switch assay (single factor experiment), one-way ANOVA analysis was performed. Results are considered statistically significant when $P < 0.05$.

Results

HS upregulates the HSF1 protein expression and induces HSF1 nuclear granules

The HSF1 protein expression significantly increased after 6h HS-exposure. Although pre-treatment with ALA did not significantly change the expression of HSF1, HSF protein levels tended to increase in heat-exposed Caco-2 cells incubated with ALA (Fig. 1A). The immunofluorescence staining revealed that exposure to HS resulted in HSF1-granule formation, whereas no clear effect of ALA treatment was observed (Figs. 1B and 1C).

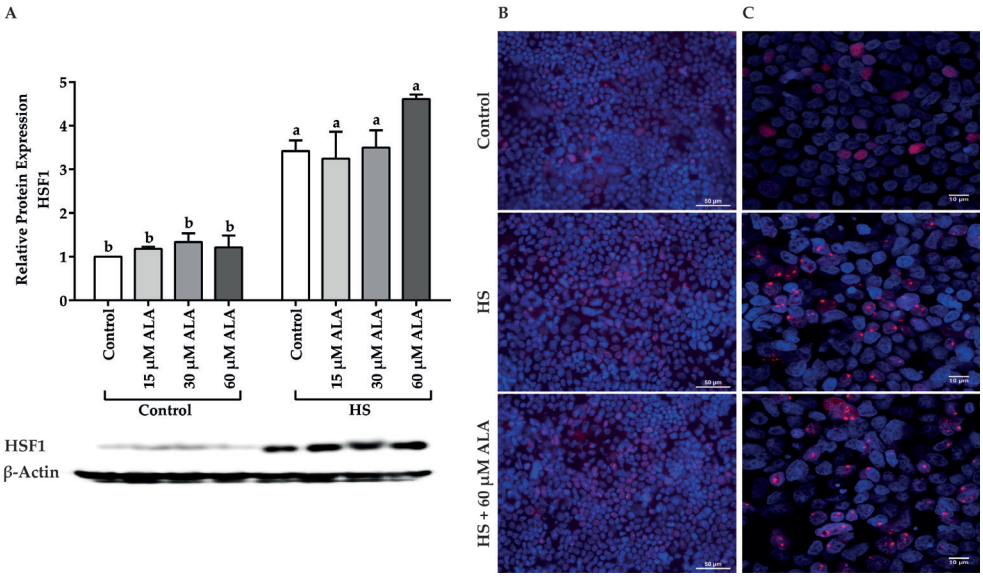


Figure 1. HS upregulates the HSF1 protein expression and induces HSF1 nuclear granules. Caco-2 cells grown on inserts and pre-treated with ALA (24h) were exposed to HS (42°C, 6h). Relative HSF1 protein expression (normalized with β-Actin) evaluated by western blot analysis (A), is expressed as mean ± SEM of three independent experiments. Different lower-case letters denote significant differences among groups. Localization of HSF1 was visualized by immunofluorescence staining. Scale bars represent 50 μm (B) and 10 μm (C).

ALA enhances the expression of HS-induced HSP70 in mRNA and protein level

Pre-treatment of Caco-2 cells with ALA enhanced the HS-induced upregulation of HSP70 in mRNA (Fig. 2A) and protein level (Fig. 2B). Significant changes were only observed at the highest ALA concentration (60 μM), whereas lower concentrations of ALA (15 μM and 30 μM) did not significantly increase the expression of HSP70 in mRNA or protein levels.

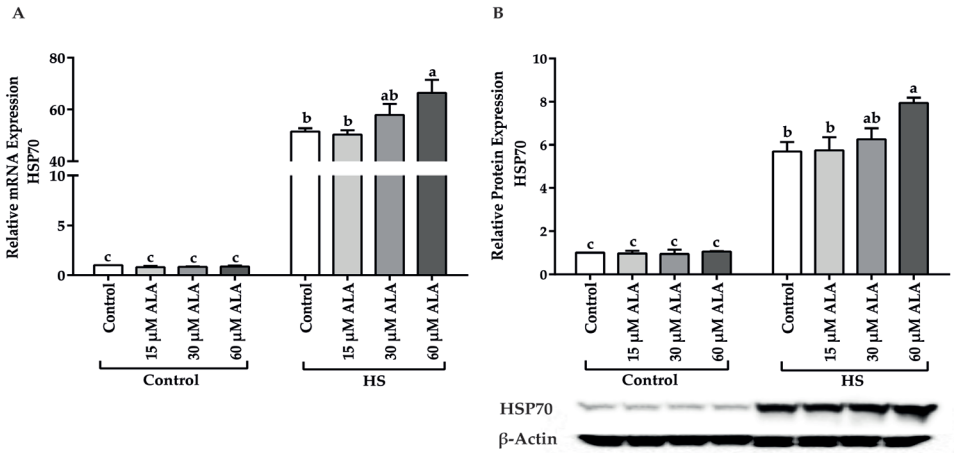


Figure 2. ALA increases the HSP70 expression under HS conditions. Caco-2 cells grown on inserts and pre-treated with ALA (24h) were exposed to HS (42°C) for 6h (qRT-PCR) or 24h (western blot) to evaluate the expression of HSP70 in mRNA (A) and protein levels (B). Results are expressed as relative mRNA expression or relative protein expression (normalized with β-Actin) as mean ± SEM of three independent experiments. Different lower-case letters denote significant differences among groups.

ALA modulated Nrf2 expression and translocation in Caco-2 cells exposed to HS

At the transcriptional level, Nrf2 was significantly upregulated under HS conditions and this effect was mitigated by ALA (Fig. 3A). Upon exposure to HS, the abundance of Nrf2 protein in the nucleus was markedly increased, and this effect was mitigated by 60 µM ALA (Fig. 3B). Exposure to ALA under control conditions slightly increased the Nrf2 protein levels in the nuclei (Fig. 3B). Moreover, ROS measurements showed that HS induced ROS generation. ALA treatment slightly, but not significantly, increased ROS levels under control as well as HS conditions (Fig. S2).

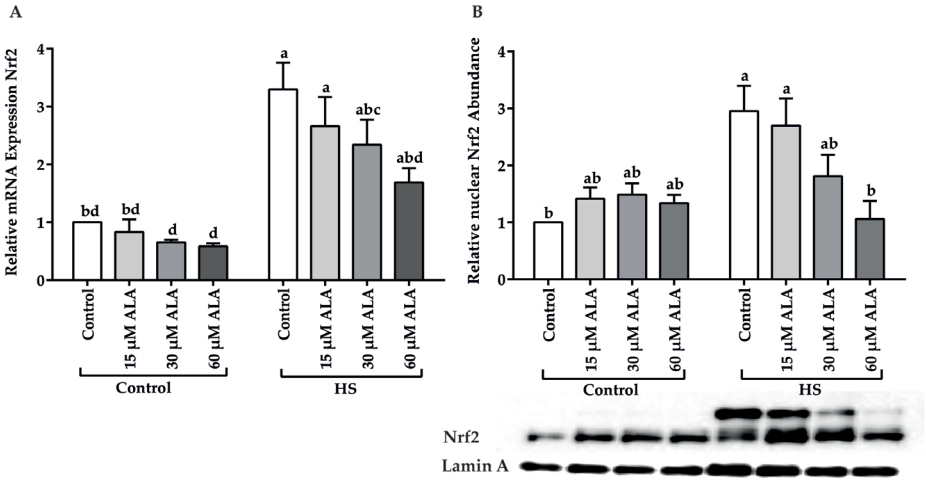


Figure 3. ALA prevents the HS-induced expression and nuclear translocation of Nrf2. Caco-2 cells grown on inserts (qRT-PCR) or 6-well plates (western blot) and pre-treated with ALA (24h) were exposed to HS (42°C) for 6h. Results are expressed as relative mRNA expression (normalized with β-Actin) (A) and relative nuclear abundance (normalized with Lamin A) (B) as mean ± SEM of three independent experiments. Different lower-case letters denote significant differences among groups.

ALA prevents the HS-induced disruption of the intestinal integrity

The effect of ALA pre-treatment on the HS-induced disruption of the epithelial barrier integrity was monitored by measuring the TEER values and LY flux. As shown in Fig. 4A, the decrease in TEER values induced by HS was significantly modulated by pre-treatment with 30 μM and 60 μM ALA. In agreement with the TEER values, the HS-induced increase in LY flux across the Caco-2 monolayer was significantly prevented by 30 μM and 60 μM ALA pre-treatment (Fig. 4B). Incubation of Caco-2 cells with 15 μM ALA, did not significantly alter the HS-induced TEER decrease and LY permeability (Fig. 4).

ALA facilitates the reassembly of junctional complexes after calcium deprivation

To examine the effect of ALA on the junctional complexes of the Caco-2 monolayer under dynamic disassembly/reassembly conditions, a calcium switch assay was performed. ALA concentrations of 30 μM and 60 μM significantly enhanced the reassembly of junctional complexes monitored by TEER measurements over a period of 8h after a temporary calcium deprivation (Fig. 4C), whereas 15 μM ALA did not have a remarkable effect (Fig. 4C).

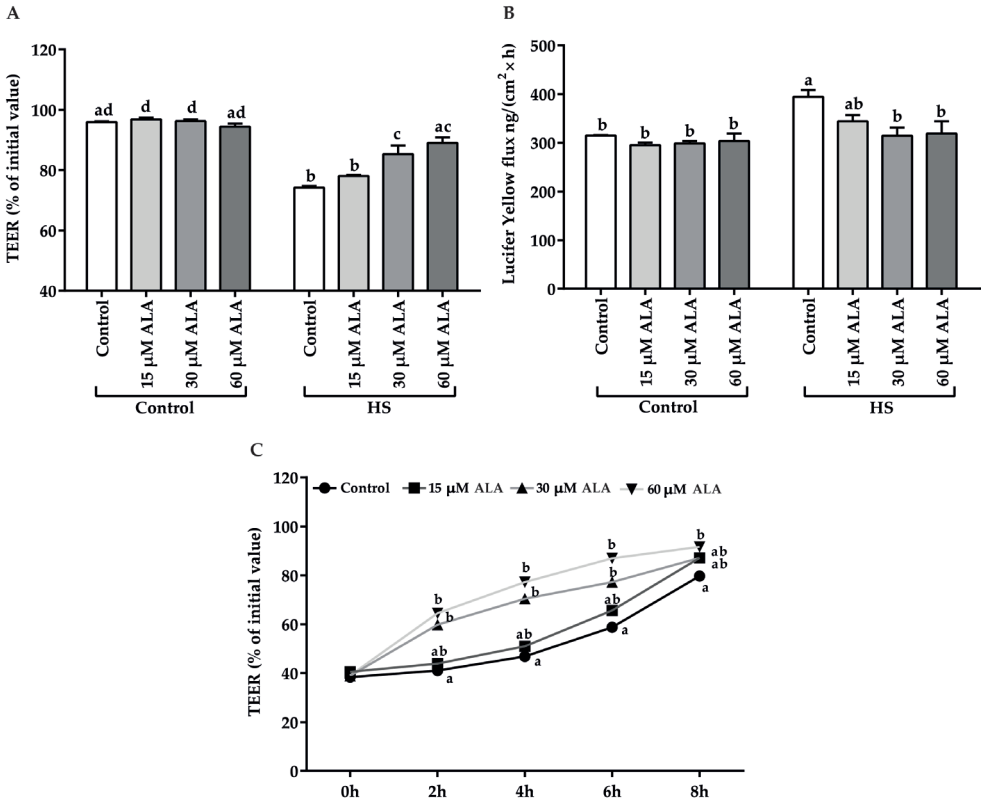


Figure 4. ALA prevents the HS-induced disruption of epithelial integrity and accelerates the junctional complexes reassembly. Caco-2 cells grown on inserts and pre-treated with ALA (24h) were exposed to HS (42°C). TEER (A) and LY transport (B) across the Caco-2 monolayer was measured after 24h exposure to HS. Data are expressed as a percentage of initial value (TEER) or in the amount LY transported [ng/(cm^2 × h)] as mean ± SEM of three independent experiments. Different lower-case letters denote significant differences among groups. Caco-2 cells were pre-treated with ALA before calcium deprivation and TEER was measured during recovery (0, 2, 4, 6, 8h) in medium supplemented with ALA (C). Different lower-case letters denote significant differences compared to control at each independent time point.

ALA partly prevents the HS-induced disturbance of E-cadherin expression

Western blot analysis showed that exposure of Caco-2 cells to HS for 24h resulted in significant decrease in E-cadherin protein level, which was partly, but not significantly, prevented by pre-treatment with the highest ALA concentration (60 µM) (Fig. 5A). The immunofluorescence staining of the E-cadherin distribution is depicted in Fig. 5B. In control cells, E-cadherin is localized at the cell membrane and appeared as belt-like structure around each cell. HS disrupted the distribution pattern of E-cadherin, whereas pre-treatment with 60 µM ALA hampered this HS-induced irregular distribution of E-cadherin.

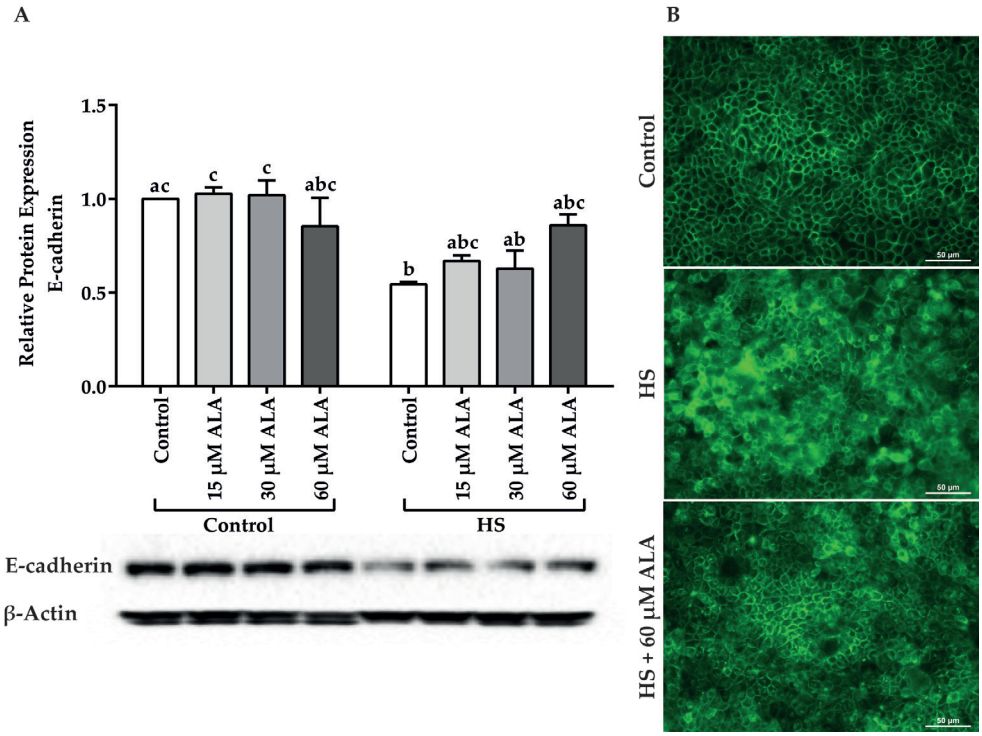
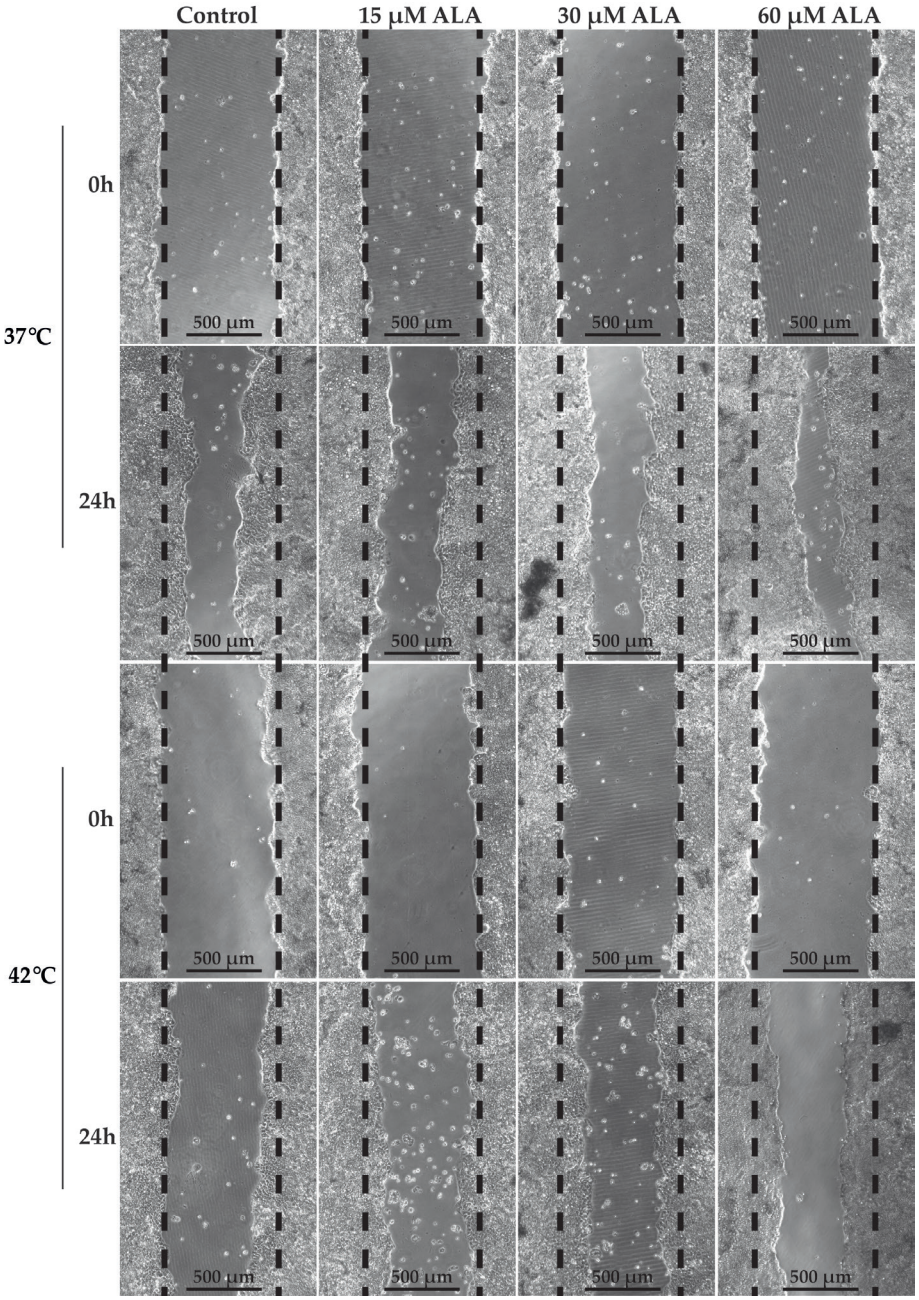


Figure 5. ALA partly prevents the HS-induced disturbance of E-cadherin expression. Caco-2 cells grown on inserts and pre-treated with ALA (24h) were exposed to HS (42°C) for 24h to evaluate the E-cadherin protein expression (A) and cellular distribution (B). For the western blot analysis, results are expressed as relative protein expression (normalized with β-Actin) as mean ± SEM of three independent experiments. Different lower-case letters denote significant differences among groups. Localization of E-cadherin was evaluated by immunofluorescence staining. Scale bars represent 50 µm.

ALA stimulates the restitution of intestinal epithelial wound healing

Pre-treatment of the Caco-2 cell monolayers with 30 µM and 60 µM ALA, significantly enhanced the wound healing at 37°C. Exposure to HS caused a slower wound area closure compared to control cells at 37°C, whereas the different concentrations ALA (15 µM, 30 µM and 60 µM) significantly stimulated wound healing under HS conditions (Fig. 6).



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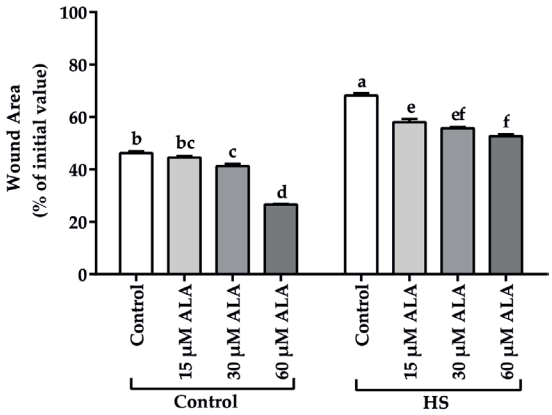

Figure 6. ALA stimulates the restitution of epithelial wound healing. Confluent Caco-2 cells grown in 6-well plates and pre-treated with ALA (24h) were scratched with a 200 µl pipette tip and were exposed to control or HS (42°C) conditions for 24h. Phase contrast images were acquired immediately after scratching (0h) and 24h thereafter. Wound widths are expressed as percentage of initial value as mean ± SEM of three independent experiments. Different lower-case letters denote significant differences among groups. Scale bars represent 500 µm.

ALA prevents the HS-induced upregulation of COX-2 and TGF-β mRNA expression

Heat treatment significantly induced the TGF-β and COX-2 mRNA expression. Pre-treatment with ALA prevented the upregulation of TGF-β (Fig. 7A) and COX-2 (Fig. 7B) in a concentration-dependent manner.

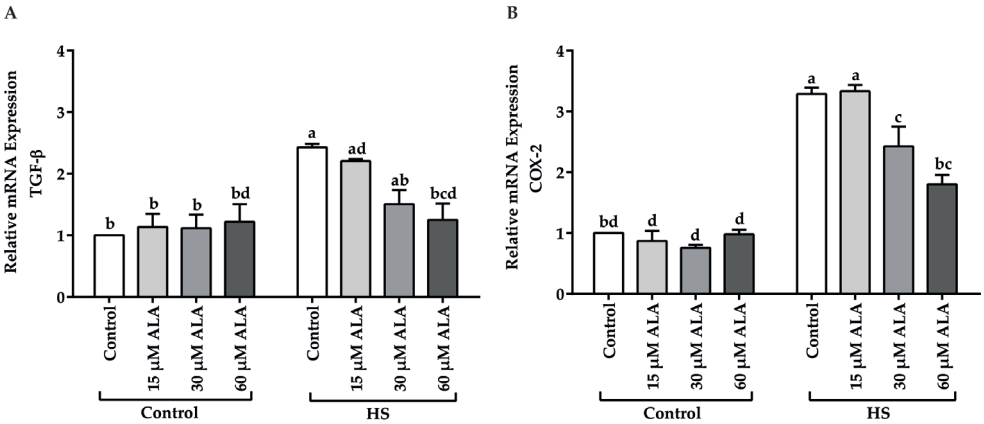

Figure 7. ALA prevents the HS-induced upregulation of TGF-β and COX-2 mRNA expression. Caco-2 cells grown on inserts and pre-treated with ALA (24h) were exposed to HS (42°C, 6h) to evaluate the mRNA expression of TGF-β (A) and COX-2 (B) (qRT-PCR). Results are expressed as relative mRNA expression (normalized with β-Actin) as mean ± SEM of three independent experiments. Different lower-case letters denote significant differences among groups.

Discussion

α-lipoic acid is considered as one of the most potent cellular antioxidants and the chemical reactivity of ALA is mainly dependent on its dithiolane ring. The oxidized and reduced ALA forms (oALA and DHLA) create a potent redox couple, which is also called the "universal antioxidant" [1]. ALA participates in regenerating other antioxidants and, unlike ascorbic acid, DHLA is not destroyed by scavenging free radicals, but can be recycled from ALA. When ALA is administered in the diet, it accumulates in several tissues and a substantial part converts to DHLA via lipomide dehydrogenase after ingestion. The cellular reduction of ALA to DHLA is accomplished by NAD(P)H-driven enzymes, such as thioredoxin reductases [5].
ALA is recommended as a potential intervention strategy for pathologies associated with oxidative stress, and also exerts anti-inflammatory properties and is known to protect the intestinal barrier [7–9,20]. We and others showed that HS can induce oxidative stress and lead to intestinal barrier disruption and inflammation [12–14]. Hence, the aim of this study was to investigate the potential beneficial effects of ALA against HS-induced intestinal epithelial injury using an *in vitro* Caco-2 cell model.
A heat shock response (HSR) is initiated by activation of HSF1 transcription factor and upregulation of HSPs to promote cell adaptation and survival under a wide range of proteotoxic stressors, including thermal stress [21]. Therefore, in the current study, the effect of HS on the expression of HSF1 and HSP70 were investigated. Our results show that under HS conditions, HSF1 and HSP70 expression levels were significantly higher in comparison with the control group. Immunolocalization of HSF1 showed granule formation in the nucleus of the cells after HS-exposure. It is described that HSF1 granules are transiently formed when heat shock genes are transcriptionally expressed, and quickly disappear after attenuation of HS induced gene transcription [21]. Under HS conditions, pre-treatment with ALA, slightly but not significantly increased the HSF1 protein expression, whereas elevated mRNA and protein expression of HSP70 were observed. Although HSF1 mediates the upregulation of HSP70, overexpression of HSP70 can negatively regulate HSF1 transcriptional activity as described by Shi *et al.* [22]. There are already some indications that ALA may modulate the HSR by inducing HSPs or HSF1 [23–25]. For example, Oksala *et al.* described an increase in HSF1 mRNA expression in the kidney of both diabetic and nondiabetic rats after ALA supplementation [23]. Furthermore, enhanced recovery levels of inducible HSP70 in the muscle of horses were observed after ALA supplementation, which may increase oxidative capacity and support tissue protection and adaptation [26]. ALA can also induce the expression of other HSPs, including HSP25 (muscle), HSP60 (liver), HSP72 (kidney, muscle), and HSP90 (kidney) [23,25–27].
The capability of ALA to modulate HSP expression has been shown to prevent oxidative injury [4]. To determine the effect of ALA on the HS-induced oxidative

stress response, Nrf2 mRNA expression as well as nuclear translocation and ROS production were measured. ALA may act as a pro-oxidant, which triggers the Nrf2-dependent transcriptional activity by forming lipoyl-cysteinyl mixed disulfides on Keap1, a protein which suppresses Nrf2 in the cytoplasm [5,28]. Our results clearly demonstrate that HS increases the mRNA expression and nuclear translocation of Nrf2. In turn, after pre-treatment with ALA, the mRNA expression and nuclear translocation of Nrf2 is decreased under HS conditions. To defeat the HS-induced oxidative stress, Nrf2 dissociates from Keap1, translocates to the nucleus and transactivates the expression of several cytoprotective genes, such as glutathione and haem oxygenase-1 (HO-1) to enhance cell survival [29,30]. In our experiments ALA treatment only slightly, but not significantly increased the HO-1 mRNA expression under HS conditions (Fig. S3).

Supporting the previously described role of ROS generation in a HS-induced oxidative stress response [31], we could show a significant increase in ROS generation induced by HS. A slight, but not significant induction of ROS was demonstrated after ALA pre-treatment under control as well as HS conditions. Although excessive ROS production is related to oxidative damage and cell death, available evidence suggests that a moderate increase in ROS levels, induced by pro-oxidants including ALA, is positively correlated with enhanced cell survival and resilience to oxidative damage [28,32]. To unravel the underlying mechanism, Jiang *et al.* showed that ALA pre-treatment in H9c2 cells attenuates cell damage through activation of Akt/Gsk-3β signalling in a ROS-related manner. Blockade of AKT activation as well as the inhibition of ROS production, abrogates the cytoprotection induced by ALA [33].

Since ROS levels were not significantly affected by ALA under HS conditions, there may be also ROS-independent pathways involved in the regulation of Nrf2. It has been suggested that different protein kinases, including casein kinase-2 and phosphoinositide-3-kinase can be involved in this ROS-independent activation of Nrf2 [34,35]. Another possible mechanism can be related to the maintenance of cellular homeostasis induced by HSP70 upregulation. Guo *et al.* showed that HSP70 significantly increased the glutathione-related enzymes to preserve the cellular redox balance [36]. However, the exact mechanisms involved in the ROS-independent regulation of Nrf2 remains to be further elucidated.

Our previous *in vitro* and *in vivo* investigations showed that induction of HS is associated with disruption of intestinal integrity, which was linked mainly to alterations in E-cadherin protein expression and localization [12,13]. This study indicates that ALA prevents the HS-induced decline in TEER levels in a concentration-dependent manner and this effect is confirmed by a reduced LY flux across the intestinal epithelial monolayer. The preventive effect of ALA against the HS-induced delocalization of E-cadherin may be related to the redox balance stabilized by ALA. It has been suggested that oxidative stress can induce a tyrosine-kinase-dependent dissociation of E-cadherin-β-catenin and occludin-ZO-1 complexes, which leads to their redistribution and disruption of barrier integrity

[37]. Another possible mechanism, is the known upregulation of HSP70, which is pivotal in preserving the barrier function under stress conditions [38]. Recently, an essential role of HSP-mediated cytoskeletal repair in a Caco-2 model of celiac disease was observed, as a shift of HSP70 from the cytoplasmic fraction into the cytoskeletal fraction of Caco-2 cells, resulted in the maintenance of barrier integrity through stabilization of E-cadherin protein [39]. Additionally, we observed that ALA supports the acceleration of the junctional complexes after calcium deprivation under thermal neutral conditions. Although the underlying mechanism is not fully understood, it has been previously reported that ALA stimulates the recovery of the intestinal epithelial architecture by increasing the mRNA and protein expression of the TJ proteins occludin and ZO-1 in a model system of post-weaning diarrhoea in rats. These findings were confirmed by *in vitro* studies with IEC-6 intestinal epithelial cells [9]. However, the mRNA and protein expression of different TJ proteins (claudin-1,-3 and -4, occludin and ZO-1) were not affected in the current Caco-2 cell model as previously described by our group [12].

HS-induced morphological damage to the intestinal mucosa is associated with villi denaturation [40]. To mimic this type of injury, we conducted a wound healing assay. As ALA accelerated the wound healing, these findings suggest that re-epithelialization of the intestinal epithelial monolayer under thermal neutral and HS conditions can be expected. In *in vivo* experiments in rats, Ma *et al.* demonstrated that supplementation of the diet with ALA improves the morphology of the small intestine damaged by glycinin [6]. Redox-regulated processes are relevant to cell proliferation and wound healing [41] and depending on the concentration, ROS is described to stimulate epithelial cell proliferation and migration by promoting the phosphorylation of ERK1/2 and the expression of cyclin D1 [42]. Furthermore, the role of induced HSP70 expression should also be taken into account in the improved wound healing process by ALA, since HSPs suppress misfolding of proteins, thereby preventing cell proliferation arrest [43].

It has previously been reported that TGF-β signalling is involved in the inhibition of epithelial cell proliferation, failure in wound healing and the process of fibrosis [44]. Therefore, the mRNA expression of TGF-β was investigated in the HS-challenged Caco-2 cells. Our results show that ALA pre-treatment was able to abolish the upregulation of TGF-β under HS conditions in a concentration-dependent manner and it can be suggested that ALA inhibits P38 mitogen-activated protein kinase (MAPK), which is involved in regulation of TGF-β mRNA expression [45].

We also investigated the modulatory effect of ALA on the mRNA expression of COX-2, as an indicator of the inflammatory responses. Rossi *et al.* showed that HSF1, induced by HS-exposure, binds to the COX-2 promoter and triggers the upregulation of COX-2 [46]. It has also been verified that oxidative stress is an important factor for the induction of COX-2 [47]. In the current study, ALA prevented the HS-induced upregulation of COX-2 mRNA expression. Considering the fact that COX-2 is a well-known nuclear factor kappa B (NF-κB) target gene [48], it is likely that ALA

treatment prevented the COX-2 upregulation through NF-κB repression [1,49].

Conclusions

With the current *in vitro* experiments it can be demonstrated that in a Caco-2 cell model, the antioxidant ALA modulates not only the HS-induced oxidative stress response, but also prevents the disruption of intestinal barrier integrity by accelerating the reassembly of junctional complexes, preventing the delocalization of E-cadherin and stimulates the intestinal epithelial healing, probably by enhancing the expression of HSP70 and preventing the fibrotic response induced by TGF-β. These *in vitro* results provide additional insights into the mechanisms of action of ALA (summarized in Fig. 8). Future *in vivo* experiments are needed to confirm the beneficial effects of dietary supplementation with ALA as a strategy to improve the resilience of animals and humans to hyperthermia-induced gastrointestinal injury.

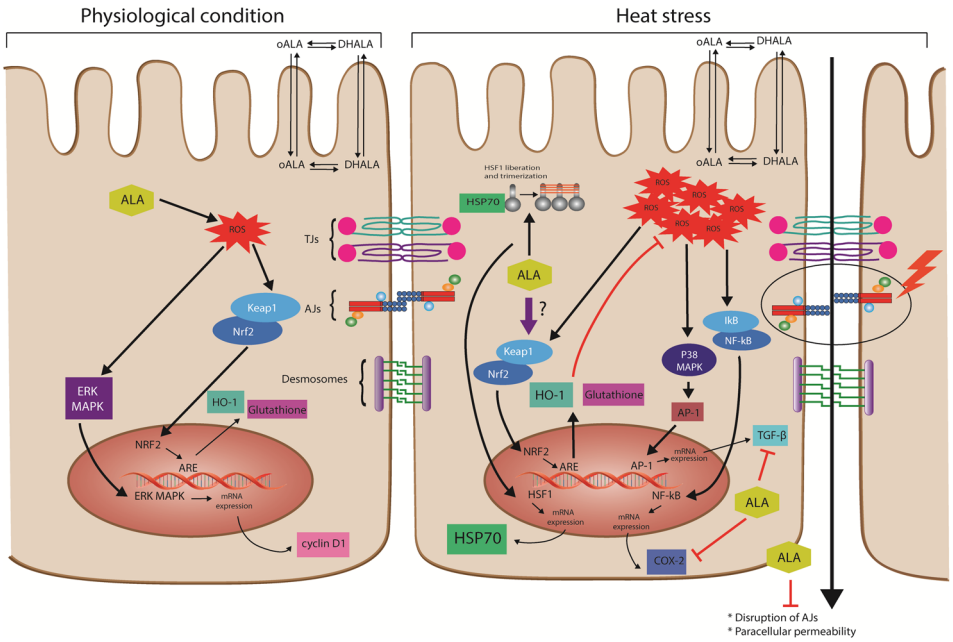

Figure 8. Proposed mechanism by which ALA regulates intestinal epithelial cells under HS conditions. Under physiological conditions, ALA induces a slight ROS production, which can trigger dissociation of Nrf2 from Keap-1 and Nrf2 translocation to the nucleus leading to the transcriptional activation of antioxidant enzymes. In addition, the ALA-induced ROS production stimulates ERK phosphorylation and its downstream pathway to promote cell proliferation (cyclin D1).
Under HS conditions, HSPs genes including HSP70, are transcriptionally activated by HSF1. ALA promotes this process, leading to preservation of cellular homeostasis and reduction in cell damage. HS is also associated with disruption of the intestinal epithelial integrity, particularly by affecting E-cadherin, which is prevented by ALA. The ROS production is increased by HS exposure and leads not only to Nrf2 liberation and nuclear translocation resulting in upregulation of cytoprotective genes, such as glutathione and HO-1. HS also stimulates the P38 MAPK and NF-κB pathways, thereby modulating the expression of TGF-β and COX-2. ALA inhibits the expression of HS-induced TGF-β and COX-2 mRNA expression. It remains to be further elucidated whether the preventive effects of ALA on the HS-induced expression and nuclear accumulation of Nrf2 is direct or indirect. Abbreviations used: AJs, adherens junctions; ALA, α-lipoic acid; AP-1, activator protein 1; ARE, antioxidant responsive element; COX-2, cyclooxygenase-2; DHALA, dihydro-lipoic acid; ERK, extracellular signal-related kinases; HO-1, haem oxygenase-1; HSF1, heat shock factor 1; HSP70, heat shock protein 70; IkB, Inhibitor of kappa B; Keap1, Kelch-like ECH-associating protein-1; MAPK, Mitogen-activated protein kinase; NF-kB, nuclear factor kappa B; Nrf2, nuclear factor erythroid 2-related factor-2; oALA, oxidized α-Lipoic Acid; ROS, reactive oxygen species; TGF-β, transforming growth factor-β; TJs, tight junctions.

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Supporting Information

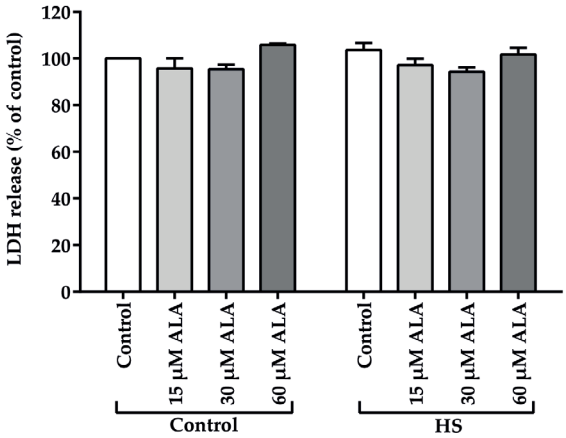

Figure S1. Neither HS nor ALA treatment exerts a cytotoxic effect on Caco-2 cells. Caco-2 cells grown on inserts and pre-treated with ALA (24h) were exposed to HS (42°C, 6h). Thereafter, the cytotoxic effect of HS (24h) and ALA treatment was determined by measuring the release of lactate dehydrogenase (LDH) in supernatants of the apical compartment of the inserts using the CytoTox 96 Non-Radioactive CytoToxicity Assay Kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. Results are expressed as relative LDH release as mean ± SEM of three independent experiments.

Table S1. Primer sequences used for qRT-PCR

	Primer sequence (5′-3′)			
Genes	**Forward**	**Reverse**	**AT**	**References**
β-Actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	63	NM_001101
HSP70	AGAGCCGAGCCGACAGAG	CACCTTGCCGTGTTGGAA	57	NG_011855.1
Nrf2	CAGGTTGCCCACATTCCCAAATCA	AGCAATGAAGACTGGGCTCTCGAT	60	NM_001145413.2
TGF-β	CACGTGGAGCTGTACCAGAA	GAACCCGTTGATGTCCACTT	60	NM_000660.5
COX-2	GGAACACAACAGAGTATGCG	AAGGGGATGCCAGTGATAGA	60	NM_000963.3

AT, Annealing temperature (°C)

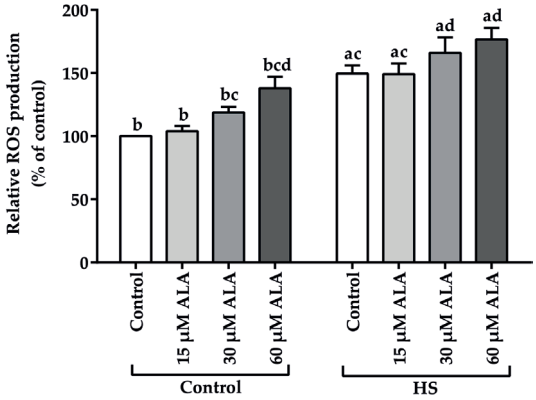

Figure S2. HS increases ROS production. Caco-2 cells grown on 96-well plate (17 days) were pre-treated with different ALA concentrations for 24h. After washing with pre-warmed PBS, cells were incubated with DMEM supplemented with 10 µM DMSO-dissolved H_2DCFDA (Life Technologies Grand Island, NY, USA) for 1h. Thereafter, cells were washed, incubated with ALA and exposed to 37°C or 42°C for 6h. Relative ROS production was quantified immediately before HS exposure and 6h after HS by a fluorometer (FLUOstar OPTIMA, Offenburg, Germany) at excitation and emission wavelengths of 490 nm and 525 nm). Results are expressed as relative ROS production normalized with initial ROS values. Data are expressed as mean ± SEM of three independent experiments. Different lower-case letters denote significant differences among groups.

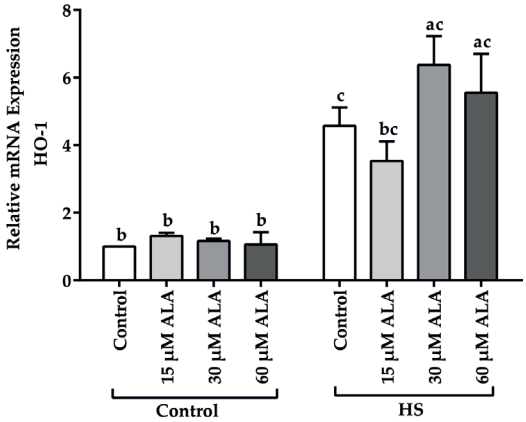

Figure S3. HO-1 mRNA expression is increased after HS exposure. Caco-2 cells grown on inserts and pre-treated with ALA (24h) were exposed to HS (42°C) for 6h (qRT-PCR) to evaluate the expression of HO-1 in mRNA level. Results are expressed as relative mRNA expression (normalized with β-Actin) as mean ± SEM of three independent experiments. Different lower-case letters denote significant differences among groups.

Chapter 8

General discussion

Under physiological conditions, core body temperature is controlled within a narrow range in order to preserve optimal physiological functions [1]. Humans and animals facing hyperthermia challenges must be able to preserve their core body temperature and maintain heat gain and heat dispersion [1]. Heat stress (HS) is typically defined as a core body temperature above 40°C [2,3]. Clinically, two forms of HS are distinguished: classical or environmental HS and exertional HS [3]. Classical HS, which occurs due to elevated environmental temperatures, is common in many parts of the world and affects particularly very young and elderly people because of the inability of their body to dissipate sufficient heat. Exertional HS results from prolonged excessive activities, such as strenuous exercise for a longer period, or at high ambient temperatures when internal heat production exceeds the capacity of thermoregulatory mechanisms of the body [4,5].
Fever, as another source of hyperthermia is considered as an important reaction of the acute phase response to infection and is denoted also as "pyrogenic hyperthermia" [6]. Although fever and HS both are characterized by an elevated core body temperature and changes in thermoregulatory responses, there are major differences in their aetiology and pathophysiology. Most importantly, during a febrile response, the hypothalamic temperature set point is elevated, whereas under HS conditions, the hypothalamic temperature set point remains unchanged [7].

Heat shock response

At the cellular level exposure to HS triggers the heat shock response (HSR) via activation of heat shock factors (HSFs), which are considered as stress integrators being involved in the synthesis of the heat shock proteins (HSPs). In mammals, HSF1 is considered as the most stress-responsive HSF, whereas HSF3 is required for this response in avian species [8,9]. HSPs act as cellular chaperones to inhibit protein misfolding and aggregation and to maintain cellular homeostasis [10]. Based on their molecular weight, structure and function, they are mainly classified into 5 different groups, including families of small HSPs (molecular weight of 15–30 kDa), HSP60, HSP70, HSP90 and HSP110, of which HSP70 and HSP90 are critically important during cell growth and are associated with the regulation of the cell cycle and the proliferative cell responses [11,12]. The overexpression of HSPs not only represent the ability of a cell to increase the resilience to stress conditions, but is also considered as a sensitive biomarker that directly represent the degree of cellular stress and damage [13,14].
The critical role of HSPs, such as HSP70 in preserving the cellular homeostasis under stress conditions, is of great interest for the development of anti-cancer strategies targeting HSP70 overexpression [15–17].

The intestines as vulnerable tissue under heat shock conditions

In response to (non-febrile) HS conditions, thermoregulatory and vascular adaptive mechanisms compromise the intestinal blood flow and redirect the blood flow

to peripheral (cutaneous) arteries in order to dissipate excessive heat. In turn, this leads to decreased blood supply in the splanchnic area followed by hypoxic/ ischemic stress conditions in the intestines [3,18]. In addition, it has been shown that under HS conditions caused by extensive exercise, the intestinal temperature is rising significantly higher than the core body temperature measured in the oesophagus [19,20]. The hypoxic and hyperthermic stress result in a disturbed intestinal homeostasis and the disruption of intestinal barrier integrity [18,20]. The key elements of the intestinal barrier are the apical junctional complexes. The junctional network comprises tight junctions (TJ) and adherens junctions (AJ), sealing the paracellular space between the adjacent cells to restrict transport of luminal antigens, toxins and bacteria across the intestinal epithelium. Under HS conditions, protein synthesis and function are impaired, and many of the clinical signs associated with HS and heat stroke are related to the loss of intestinal integrity. A comprehensive summary of the current understanding of the effects of HS on intestinal integrity and function is presented in **Chapter 2**.

***In vitro* models for the evaluation of adverse effects of HS on intestinal integrity**

Human colonic carcinoma cell lines, such as Caco-2 and T-84, are the well-studied cell lines used for the evaluation of the adverse effects of HS on intestinal homeostasis. Although both Caco-2 and T-84 cells get differentiated into a tight intestinal epithelial monolayer and form cell-cell contacts with junctional complexes [21], Caco-2 cells are generally preferred, as they develop well-defined brush borders, and are validated as a useful *in vitro* model system for intestinal epithelial permeability [22]. Additionally, considering the fact that HS mainly affects the integrity in the small intestine in different *in vivo* models [23–25], Caco-2 cells, although originally derived from a colon carcinoma, resemble structurally and functionally the cells of the small intestine and seem to be a more appropriate *in vitro* model compared to T-84 cells, resembling colonic crypt cells [21]. With the aim to describe the HS effects on the TJ complex in more detail, Dokladney *et al*. showed that exposure of Caco-2 cells to elevated temperatures (39-41°C/ 24h) resulted in an upregulation of occludin and downregulation of ZO-1 protein expression [26]. In **Chapter 3** of the current thesis, a comparable approach is chosen. In this Chapter we describe an *in vitro* model established to investigate the adverse effects of elevated temperatures on the epithelial integrity, using differentiated Caco-2 cells grown as a monolayer on transwell inserts. Exposure of the cell monolayers to HS (42°C/ 24h) significantly increased the E-cadherin mRNA expression. In contrast, the protein concentration of E-cadherin was decreased and an irregular redistribution of E-cadherin was observed, while TJ proteins remained unaffected [13]. Different heat exposure times, temperatures and recovery periods may result in different expression levels of TJ and AJ proteins. Therefore, E-cadherin can be recognized as an important target in HS-induced disruption of cell-cell contacts [24,27]. Proteomic investigations in the small intestines of rats exposed to HS showed that Myosin Heavy Chain 9

(Myh9), a regulator of cadherins and a major cytoskeleton protein that binds to the F-actin cytoskeleton to regulate cellular migration, adhesion and maintenance of cell shape, was downregulated after HS [28]. In addition, secreted HSP90 is known to induce Nuclear Factor-κB (NF-κB)-mediated TCF12 protein expression, which is recognized as transcriptional repressor of E-cadherin [29].

Heat-induced oxidative stress

Exposure to HS directly and indirectly (via induction of hypoxia) also results in a disturbed balance between reactive oxygen species (ROS) and the antioxidant defence system leading to oxidative stress [30]. Oxidative stress can activate the nuclear factor erythroid 2-related factor-2 (Nrf2) pathway to increase the expression of antioxidant enzymes, including haem oxygenase-1 (HO-1), superoxide dismutase and glutathione peroxidase, which maintain the cellular redox balance [31,32]. HSF1 and Nrf2 transcription factors engage in crosstalk for cytoprotection by controlling overlapping transcriptional targets, such as HO-1, and they may compensate for each other [32]. Hensen *et al*. reported that cells with dysfunction of HSF1 due to a mutation are still capable to upregulate HSP70 and HO-1 under HS conditions, although with delay. This response indicates that Nrf2 can modulate the HSR independent from HSF1 [33]. Our investigations in **Chapter 3** and **Chapter 7** clearly show that HSP70 and HO-1 were both transcriptionally activated by elevated temperatures in Caco-2 cells.

We (**Chapter 3** and **Chapter 4**) and others showed that the protective effect of some health-promoting substances, such as prebiotics and radical scavengers, which attenuate oxidative stress, also result in suppressed expression of HSPs in different cell lines [13,24,34,35], On the other hand, the beneficial effects of some health-promoting molecules are strategically related to enhancement of HSPs expression [36–38] (also demonstrated in **Chapter 7**).

Intervention strategies to attenuate the adverse effects of HS on the cellular homeostasis and barrier integrity in intestinal epithelial cells

Considering that disruption of intestinal barrier integrity resulting in penetration of antigens and bacterial toxins, like lipopolysaccharides (LPS), is a major aetiological factor in fatal cases of severe HS, studies have focused on the nutritional strategies to maintain the gut homeostasis and to limit the adverse effects on the intestinal barrier.

L-Arginine: non-essential, but of vital importance

Early studies showed that protein synthesis is markedly altered by hyperthermia [39–41]. Therefore, in **Chapter 6**, we hypothesize that supplementation of cell culture medium with L-Arginine (L-Arg) could have beneficial effects under HS conditions. L-Arg is considered as a "conditionally essential" amino acid. Although cells can produce basic amounts of L-Arg via protein breakdown and conversion

of other amino acids, L-Arg can be depleted during catabolic stress conditions [42]. Moreover, L-Arg is the main precursor of nitric oxide (NO) involved in multiple cellular signalling pathways in enterocytes, including intestinal immunity [43,44].
To demonstrate the essential role of L-Arg, in **Chapter 6**, cells were cultured in L-Arg free medium, hence depending exclusively on the endogenous synthesis of L-Arg. L-Arg depletion resulted in a sharp induction of the stress markers HSP70 and HO-1 under HS conditions, which was much more pronounced than "control" cells, cultured under standard conditions with cell culture medium containing 0.4 mM L-Arg. Supplementation with the maximal non-cytotoxic L-Arg concentration of 4 mM moderately upregulated HSP70, and diminished the epithelial barrier damage observed by a stabilized Trans Epithelial Electrical Resistance (TEER) and a reduced Lucifer Yellow (LY) permeability. Related to the role of L-Arg as a nitric oxide (NO) donor, it can be assumed that the protective effects of increased L-Arg availability are partly related to an increase in the cellular NO-level. Counteracting this NO-production by addition of L-NAME, the prototypic inhibitor of the NO-synthase (iNOS), we observed indeed a lower efficacy of 4 mM L-Arg regarding the preservation of TEER levels and preventing the paracellular flux of LY. Corresponding results were obtained for the E-cadherin protein expression and localization under HS conditions. These results were in line with the observation from Beutheu *et al*. who showed that L-Arg supplementation would reduce the damage to the TJs, thus preventing the disruption of intestinal epithelial integrity induced by methotrexate [45]. In addition, the beneficial effects of L-Arg on intestinal epithelial integrity and immune regulation were obliterated in iNOS-deficient mice suffering from colitis [46]. *In vivo* investigations by Gokin *et al.* demonstrated that exogenous L-Arg rescued the villous re-epithelialization following acute mucosal injury by increasing iNOS-derived NO, and iNOS inhibition abolished the intestinal repair after injury [47]. Similarly, Rhoads *et al.* showed that the beneficial effects of L-Arg in wound healing are mediated by the NO pathway and focal adhesion kinase (FAK) [48]. More recently, the same group demonstrated that in addition to the NO pathway, mammalian target of rapamycin (mTOR) signalling is also essential for intestinal wound healing [44]. Furthermore, the effect of NO in the amelioration of the intestinal integrity disruption is known to associate with the prevention of protein tyrosine phosphorylation of key TJs and AJs [49,50].

In clinical practice, patients (pre-term infants) suffering from necrotizing enterocolitis (NEC), show low serum levels of L-Arg, and hence therapeutic protocols have been developed to use L-Arg as protective agent against NEC [51,52] and recent investigations have demonstrated that administration of L-Arg will be effective in reducing the incidence of NEC in patients at risk [53,54].

α-lipoic acid (ALA): the Janus Antioxidant

ALA has been used in the treatment of different pathologies associated with redox imbalances, including diabetes, ischemia-reperfusion injury and heavy metal poisoning [55–57]. Effects of ALA has been assumed to be partly related to activation of HSR, as ALA can induce the expression of HSPs, including HSP25 (muscle), HSP60 (liver), HSP72 (kidney, muscle), and HSP90 (kidney) [37,58–60].

As a main mechanism of action, ALA exerts mild pro-oxidant properties at low concentrations, thereby stimulating the ROS production and initiating a potent antioxidative response starting with the dissociation of Nrf2 from its cytoplasmic repressor Kelch-like ECH-associated protein 1 (Keap1) and its translocation to the nucleus, where it enhances the expression of antioxidant proteins and enzymes. Under stress conditions, which are associated with the generation of high amounts of ROS, Nrf2 abundantly accumulates in the nucleus.

Since hyperthermia is known to be associated with the redox imbalance, we tested the potential beneficial effects of ALA on heat tolerance of intestinal epithelial cells (**Chapter 7**). Our results demonstrate that treatment of Caco-2 cells with ALA under thermal-neutral conditions slightly increase the nuclear Nrf2, whereas HS exposure sharply increase the mRNA expression and nuclear translocation of Nrf2. This effect can be attenuated by ALA pre-treatment, suggesting that ALA had initiated a higher antioxidant reserve as mentioned above. The sharp upregulation and translocation of Nrf2 reflects the cellular defence strategy against HS-induced oxidative stress [31,61].

Moreover, our results show that stabilization of the redox balance by ALA pre-treatment, significantly reduce the disruption of intestinal integrity followed by HS, which was observed by increased TEER values, decreased LY flux, increased expression as well as recovered distribution of E-cadherin. It is known that oxidative stress may induce a tyrosine-kinase-dependent dissociation of E-cadherin-β-catenin and occludin-ZO-1 complexes, leading to loss of barrier integrity [49]. Previous experiments revealed that ALA can decrease the intestinal permeability by increasing the expression of occludin and ZO-1 TJs in *in vitro* and *in vivo* models of oxidative stress [62]. In a murine model of ulcerative colitis, it was shown that ALA treatment will increase the expression of occludin to ameliorate the ulcerative colitis-induced intestinal mucosal damage [63].

In addition, stimulation of the HSR and increased HSP70 induced by ALA (as also demonstrated in our study), can be involved in the maintenance of intestinal epithelial integrity under HS conditions. Yang *et al.* demonstrated that pre-treatment of T-84 cells with HSP70 prevented HS-induced intestinal epithelial hyperpermeability in a concentration dependent manner [64]. Another recent *in vitro* investigation showed that HSP-mediated cytoskeletal repair in a Caco-2 model of celiac disease was related to stabilization of junctional proteins, including E-cadherin, by HSP70 resulting in increased barrier integrity [65].

In our study (**Chapter 7**) we also observed in the so-called "wound healing" model

that ALA increased wound repair. A slight ROS induction is known to stimulate the phosphorylation of ERK1/2 and the expression of cyclin D1 [66], which can explain this effect. Moreover, ALA hampers the HS-induced upregulation of transforming growth Factor-β (TGF-β) mRNA, thereby counteracting the inhibition of cell proliferation arrest induced by TGF-β [67]. This effect of ALA is assumed to be related to the inhibition of P38 mitogen-activated protein kinase [68]. Of clinical importance is the fact that ALA is capable to regulate other inflammatory cascades as well, since it prevents the upregulation of cyclooxygenase-2 a distinguished NF-κB target gene, which is known to be stimulated under oxidative stress conditions [69–71].

Taken together, ALA supplementation not only regulates the epithelial cell resilience (HSR and Nrf2) to oxidative damage induced by HS, but also can play a role in maintaining the intestinal integrity and modulating the inflammatory complications followed by HS.

Enhancing the intestinal integrity by non-digestible oligosaccharides

Non-digestible oligosaccharides like galacto-oligosaccharides (GOS) are known as functional food ingredients. Initially, it was assumed that GOS exert their protective effects through promoting the increase of beneficial bacteria in the gut, such as *Bifidobacterium* and *Lactobacillus* spp. However, recent findings have highlighted their direct, microbiota-independent effect on intestinal barrier integrity and immune homeostasis [72–75]. Based on these findings, we included GOS in the line of substances that may be recommendable to be used under HS conditions.

In **Chapter 3** the direct effect of GOS pre-treatment was evaluated in the Caco-2 cell monolayers exposed to HS. Our results show that GOS pre-treatment not only reduce the HSR, but also prevents the HS-induced disruption of intestinal epithelial integrity by preserving the E-cadherin levels. Previously, investigations from our group already demonstrated that GOS facilitate the reassembly of junctional complexes (TJs and AJs) after calcium deprivation in Caco-2 monolayers [72]. Considering that on one hand the involvement of E-cadherin, as a major component of junctional complexes in forming and stabilizing the cell-cell contacts, is Ca^{2+} dependent, and on the other hand GOS is able to maintain the expression and localization of E-cadherin (observed in **Chapter 3**), it may be speculated that this acceleration and assembly of the junctional complexes is at least in part attributable to the stabilisation of the E-cadherin expression. Bhatia *et al.* demonstrated that GOS may be directly involved in the upregulation of trefoil factor-3 (TFF3) in human adenocarcinoma cells [76] which is involved in enhancement of TJ barrier integrity [77].

Despite these various encouraging results, the molecular mechanisms involved in the microbiota-independent effects of GOS are still incompletely understood. It is expected that the size and structure of oligosaccharides are a determinative factor in their direct modulatory effects [78]. Structure-based evaluation of

oligosaccharides available in the GOS mixture showed that some oligosaccharides with lower degree of polymerisation (DP), such as DP-2 and DP-3, seem to be more effective in regulating the intestinal integrity [79]. Moreover, it is known that non-digestible oligosaccharides like GOS, may induce the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), which regulates the anti-inflammatory peptidoglycan recognition protein 3 (PGlyRP3) and GOS may interact with different receptors including carbohydrate receptors, such as C-type lectin and TLR-4, which may contribute to the protective effects of GOS *in vivo* [73,80,81]. In addition, preliminary findings from our group showed that direct anti-inflammatory effects of GOS may be due to its modulatory effect on p38 MAPK pathways observed in Caco-2 monolayers challenged with mycotoxins.
Furthermore, there may be a similar function between GOS and trehalose-based oligosaccharides, which act as antioxidants and are capable to prevent protein carbonylation. Trehalose is also known to stabilize the structure of lipid bilayers and of proteins to prevent protein aggregation (oxidative) changes in large molecules [82,83].

In **Chapter 4**, *in vivo* experiments in chickens are described that were designed to analyse the effect of a dietary GOS supplementation on HS-induced alterations, including HSR, intestinal integrity, intestinal inflammatory reactions and oxidative stress. The chickens were exposed for several days to controlled HS (38–39°C, 8h per day/ 5 days) [24] and detrimental effects of HS on gene expression of stress markers (HSF1, HSF3, HSP70, HSP90 and hypoxia-induced factor-1) were observed particularly in the ileum and to a lesser extent in the jejunum. In the chicken jejunum, all HS-induced changes (HSFs, HSPs, E-cadherin, TJs, TLR-4, IL-6 and IL-8) were suppressed by the GOS-supplemented diet, while GOS supplementation failed to modulate these markers in the ileum. A possible explanation for these distinct effects of GOS in jejunum and ileum, may be the differences in the microbiota composition in the different compartments of the intestines. GOS, like other prebiotics, are known to increase the population of *Lactobacillus* spp., which can exert intestinal barrier preserving effects and promotes anti-inflammatory mechanisms [84,85]. *Lactobacillus* spp. compose more than 99% of all species of the microflora population in the jejunum, whereas in ileum *Lactobacillus* spp. constitute 70% of microflora population [86,87]. The difference in the composition of microbiota alongside the intestine, will also result in varying microbial-HSPs (exogenous HSPs), which are able to mediate anti-inflammatory reactions in different inflammatory disease models [38,88]. In a previous study from our group it could be demonstrated that dietary GOS supplementation significantly increased the short chain fatty acid production, including butyrate, in piglets [84]. Butyrate is known to preserve the gut barrier function and shows anti-inflammatory effects in the gastrointestinal tract [89,90]. Moreover, butyrate has been shown to decrease intestinal permeability in both *in vitro* and *in vivo* studies [89,91]. Mechanistic studies show that butyrate treatment

increase AMPK activity and accelerate the assembly of TJs [92]. Furthermore, it has been demonstrated that butyrate pre-treatment reduces the phosphorylation of NF-κB P65, P38 MAPK and ERKs in damaged tissues [90]. Another study by Liu *et al.*, revealed that butyrate would enhance the recovery of gastric lesions by attenuating the contents of malondialdehyde (MDA) and carbonyl proteins as well as by decreasing the pro-inflammatory cytokines, such as IL-6, TNF-α, IL-1β [90]. Studies in patients with ulcerative colitis have shown a diminished capacity of the intestinal mucosa to oxidize butyrate [93,94]. Nancey *et al.* showed that butyrate oxidation can be reduced by TNF-α at concentrations found in inflamed human mucosa [95].

These observations show that in addition to direct effects in regulating immune responses and intestinal integrity, GOS stimulate the population of probiotic bacteria and short chain fatty acids to regulate the intestinal homeostasis. It is indicated that GOS often escape digestion in the upper parts of the intestine and are (partly) fermented in the lower parts of the intestine [96]. In addition, oligosaccharides are not fully fermented in the intestines and small amounts of human milk oligosaccharides pass through the intestinal barrier as detected in plasma and urine of breastfed infants [97]. Our group recently showed that dietary oligosaccharides are detectable in the plasma, urine and faeces of the piglets fed with a GOS-supplemented diet [98]. Hence, additional effects of the systemic role of GOS in humans and animals await further investigations.

Modulation of intestinal nutrient transporters by GOS: evidence for their involvement in the pathophysiology of HS

Nutrient transporters present in the brush border of the intestinal epithelium are primarily responsible for the uptake of nutrients and electrolytes. Their expression and function is not only affected by the amount of feed intake, feed composition and malnourished conditions, but increasing evidence suggest their involvement in the pathophysiology of intestinal hypoxic and inflammatory disorders [99,100]. **Chapter 5** focus on the expression of different intestinal brush border nutrient transporters in jejunum and ileum of broiler chickens involved in the experiments described in **Chapter 4**. In this *in vivo* experiment, exposure to HS is associated with an increase in the pro-inflammatory cytokines in the jejunum and ileum, whereas GOS counteract these effects in the jejunum (**Chapter 4**). Moreover, in **Chapter 5**, HS results in a downregulation of Liver-expressed antimicrobial peptide (LEAP-2), which is partly prevented by dietary GOS. LEAP-2 is part of the complex system of antimicrobial peptides, which are expressed in different (chicken) epithelial tissues, such as the lung and the intestines [101]. Antimicrobial peptides are important components of the host's innate immune response protecting the body against invading pathogens [101]. In the current study, It can be speculated that the stabilisation of LEAP-2 expression by GOS improves the resilience of chickens to intestinal infections [102].

Furthermore, results in **Chapter 5** show that the peptide transporter (PepT-1) is significantly upregulated in jejunum and ileum of broiler chickens exposed to HS. Previous investigations also described an increase in the expression of PepT-1 in intestinal inflammatory disorders, such as inflammatory bowel diseases (IBD) [103]. Considering the broad substrate specificity of PepT-1 to almost all di- and tripeptides, such as peptidomimetic drugs and bacteria-derived peptides, it is speculated that increased PepT-1 expression followed by intestinal inflammation, may lead to enhanced transport of bacterial products, such as the peptide structure *N*-formylmethionylleucyl-phenylalanine (fMLP), which will exacerbate the intestinal inflammation [103,104].

Dietary supplementation with GOS attenuated the HS-induced upregulation of Pep-T1 and this mechanism may contribute to the anti-inflammatory properties of GOS. The direct anti-inflammatory effects of GOS have been reported in various studies from our group. First, peripheral blood mononuclear cells (PBMCs) derived from GOS-treated foals, challenged with lipopolysaccharides, result in downregulated immune responses, observed by lower interferon-γ and IL-6 expression [105]. Second, it has been shown that GOS treatment attenuated the inflammatory responses after a challenge with the mycotoxin Deoxynivalenol in human Caco-2 cells and $B6C3F_1$ mice [72].

The nutrient transport system in the intestinal epithelium is a highly O_2 and energy-dependent procedure. In response to the repartitioning of the blood volume under HS conditions, hypoxia in the intestines may induce changes in the function of intestinal epithelial transporters related to the limited availability of O_2 and ATP [18,25]. A prominent example for such adaptive changes are the sugar transport systems [100]. In the current *in vivo* study with chickens, we observe an upregulation in the mRNA expression of GLUT-5 sugar transporter following HS conditions, while SGLT-1, having an ATP-dependent function in absorption of glucose, remained unaffected. Although GLUT-5 is a fructose transporter and has a low efficacy in the uptake of glucose from the intestinal lumen, the GLUT-5 upregulation can be interpreted as a compensatory response to the reduced SGLT-1 function under hypoxic conditions. Dietary GOS also significantly prevent the HS-induced upregulation of GLUT-5 in the chicken jejunum.

Clinical relevance

Humans

HS is a life-threatening condition for humans [5]. The highest incidence of heat-related deaths occurs in very young or elderly individuals during summer or heat waves. However in young healthy individuals, HS is also experienced following strenuous physical activity in hot climates [106]. Progression of HS to heat stroke is considered as a medical emergency with mortality approaching 10% to 30% [107]. Multiple-organ failure is considered as the end stage of heat stroke conditions affecting brain, liver, kidney and the central nervous system. Gut-derived (endo) toxins and the translocation of pathogenic bacteria are considered as the motor of this multi-organ failure and the mortality in heat stroke patients [108].

Farm animals

FAO statistics in 2010, reported that 50% of world meat and 60% of world milk originate from tropical zones with a higher risk of exposure to HS. In turn, HS has become an important challenge affecting health and welfare of farm animals, including poultry, pigs and cattle [109]. Various investigations were conducted in chickens and showed their lack of efficient thermoregulatory mechanisms. Particularly the modern poultry genotypes are one of the most susceptible animal species to high environmental temperatures [110]. Exposure of broiler chickens to HS is associated with multiple pathophysiological alterations, such as immune dysregulation, gut barrier dysfunction and cellular oxidative stress. Due to their apparent sensitivity and the still increasing poultry meat production worldwide, broiler chickens are considered as a suitable *in vivo* model to study the pathophysiology of detrimental effects in environmental HS [110–113]. In addition to poultry species, pigs are also highly vulnerable to HS, since they lack functional sweat glands and they produce a large amount of metabolic heat. To reduce metabolic heat production, pigs voluntarily reduce feed intake, which leads to limited nutrient availability and hence loss of body weight [114]. Finally, (HS-induced) nutrient restriction can lead to alterations in intestinal homeostasis and morphology, increasing the risk of dysbacteriosis and infectious diseases [25,115].
Ruminants and in particular dairy cattle, which are partially or completely reared outdoors are susceptible to exposure to environmental HS. Under HS conditions, cattle experience reduced feed intake and considerable amounts of body weight loss, resulting in poor meat quality and a remarkable reduced milk yield, and an undesirable decrease in the milk protein content. [109,116,117]. In consideration of this undesirable effect of HS on animal wellbeing, health and performance, various research programs have been initiated to identify pharmaco-dietary intervention strategies to mitigate these adverse effects, and some promising examples of dietary supplements with a potency to prevent HS-induced injuries are described in this thesis.

Conclusions

With the prospect of global warming, which increases the exposure of humans and animals to HS, studying the adverse effects of HS on the gut homeostasis has become a hot topic.

The relation between HS (environmental or exertional) and gut dysfunction is complex and multifactorial. However, it seems that hyperthermia not only shifts the blood flow from the visceral to the peripheral circulation to enhance heat dissipation resulting in hypoxia and oxidative stress in the intestines, but also directly affects the cell-cell contacts in the intestinal epithelium leading to a disruption of the intestinal epithelial barrier. Different *in vitro* and *in vivo* investigations in recent decades highlight the vulnerability of junctional complexes to hyperthermia. As a result, penetration of allergens and (bacterial) toxins into the submucosal tissues exaggerates the inflammatory response and exacerbates epithelial damage. Future research should focus on the relation between HSPs and the cytoskeleton and their effect on stabilizing the junctional complexes as well as on the molecular pathways involved in the alterations in the expression and assembly of different TJs and AJs under HS conditions.

Although different promising candidates for a pharmaco-nutritional intervention against HS-induced intestinal injuries have been identified as discussed above, further investigations are needed to optimize their use in humans and individual animal species. Therefore, further *in vivo* studies are warranted to increase our knowledge and understanding of the complex HS response and adaptation to hyperthermia and its impact on gut health.

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Chapter 9

General Summary

Climate changes have increased the frequency and intensity of environmental and exertional heat stress (HS) conditions. Unabated HS overwhelms the homoeothermic regulation and affects the function of different organs, leading to a syndrome of multi-organ dysfunction. However, investigations in the last 3 decades have shown that systemic inflammation due to intestinal dysfunction induced by HS has to be considered as the pivotal key event in hyperthermia-related disorders.
In vivo, the association of HS and gut barrier dysfunction is mainly related to the thermoregulatory mechanism of the body to shift the blood flow from internal organs to peripheral circulation to facilitate heat dissemination. In turn, insufficient supply of oxygen and nutrients to the intestines triggers oxidative stress, protein denaturation and intestinal barrier breakdown.
Several endogenous defence mechanisms can be activated to limit (heat) stress-induced deleterious effects and increase the tolerance under stressful conditions. The main resilience pathway is the multi-faceted heat shock response (HSR) involving several transcription factors and signal transduction pathways. Specific heat shock proteins (HSPs) are the main functional members of the HSR and are involved in the prevention of protein aggregation and misfolding, promoting the return of damaged proteins into native conformations, thus maintaining protein homeostasis. An inadequate HSR or failure/dysfunction of cell resilience pathways due to the intensity of HS lead to damaged intestinal epithelial barrier integrity and a severe inflammatory response that may progress into an often fatal heat shock. In recent years, nutritional strategies are introduced as potential avenues to enhance the resilience to HS conditions in animals and humans being at high risk of environmental or exertional HS.
In consideration of the increasing prevalence of HS conditions, the aim of this thesis was:

I) To determine the cellular series of events involved in the HSR in intestinal epithelial cells, using *in vitro* and *in vivo* HS models, including colorectal adeno-carcinoma (Caco-2) cell monolayers and broiler chickens, respectively.
II) To test nutritional supplements of different classes, which can modulate the adverse effects of HS on the intestinal barrier function.

In **Chapter 2** of this thesis, the vulnerability of the intestinal epithelial barrier to HS exposure is discussed and the main cellular adaptation mechanisms involved in the cell resilience to stress conditions, including HS, have been reviewed.
Considering that leakiness of intestines in response to HS may be associated with penetration of luminal antigens, endotoxins and bacteria into the blood stream leading to intestinal and systemic inflammatory responses, it is essential to improve gut homeostasis and barrier integrity during HS. Therefore, in this chapter we introduced also some nutritional interventions, which have a potency to interfere with the adverse effects observed during HS conditions, including hypoxia,

disruption of the intestinal barrier integrity and inflammation.

In **Chapter 3**, an *in vitro* epithelial Caco-2 cell model is established to assess the effects of hyperthermia on the expression of HSPs as well as on the intestinal barrier function. Moreover, the direct (microbiota-independent) effects of galacto-oligosaccharides (GOS) in maintaining the intestinal epithelial integrity are tested. Results showed that elevating ambient temperatures (40 and 42°C, 24h) increase HSP70 and HSP90 mRNA and protein expression and induce an upregulation in haem oxygenase-1, a marker of oxidative stress. Additionally, exposure to HS is associated with a disruption of intestinal integrity measured by a decreased Trans Epithelial Electrical Resistance (TEER) and increased paracellular permeability of the marker Lucifer Yellow (LY). Although HS exposure did not change the expression of tight junction proteins, E-cadherin mRNA expression increased significantly. However, investigations on the protein expression of E-cadherin revealed that HS markedly downregulates the E-cadherin protein expression. Pre-treatment with GOS, reduces the HSR and preserves the intestinal epithelial integrity by preventing the heat-induced effects on E-cadherin expression and distribution.
The results in this study confirm the microbiota-independent effects of GOS on preserving the intestinal integrity and modulating the response to HS. Thus, GOS may be an attractive dietary supplement for individuals being at risk to develop HS.

In **Chapter 4**, we describe an *in vivo* experiment, which was performed to study the effects of HS on intestinal homeostasis in broiler chickens. Chickens were fed a diet supplemented with 1% or 2.5% GOS (6 days) prior to and during a temperature challenge for 5 days (38–39°C, 8h per day). Different parameters of the HSR, intestinal integrity, pro-inflammatory cytokines and hypoxia-related markers were chosen to evaluate the effect of HS on the intestines of broiler chickens. This study showed that HS mainly affected the jejunum and the ileum in the intestines of chickens. HS resulted in an upregulation of HSF3, HSP70, HSP90, E-cadherin, claudin-5, ZO-1, TLR-4, IL-6 and IL-8 in jejunum and ileum. These HS-related effects in the jejunum were successfully prevented in chickens fed a GOS diet, while dietary GOS did not alter these effects in the ileum due to the severity of the HS-induced detrimental effects.
These results provide for the first time evidence for differences in susceptibility to HS along the chicken intestines. The results also confirmed the previous *in vitro* results described in **Chapter 3** indicating that dietary GOS indeed can play a role in stabilizing the intestinal integrity and preserving the intestinal homeostasis, particularly in the jejunum of HS-exposed broilers.

In **Chapter 5**, we present additional results obtained with intestinal tissue material from the chicken experiment described in **Chapter 4**. Here, we investigate the alterations in the function of brush border nutrient transporters, including intestinal

peptide transporter (PepT-1), glutamate/aspartate amino acid transporter-3 (EAAT-3), sugar transporters, such as the fructose transporter (GLUT-5) and the sodium-dependent glucose transporter 1 (SGLT-1) in the HS exposed chickens. Our results demonstrate that HS causes an upregulation in the mRNA expression of PepT-1 and GLUT-5 in jejunum and ileum of chickens. The GOS-supplemented diet significantly attenuated the HS-induced upregulation of PepT-1 and GLUT-5 in chicken jejunum. Additionally, in this chapter the effect of HS is examined on the expression of liver expressed-antimicrobial peptide-2 (LEAP-2), which has been recognized as key mediator of the innate host defense in the small intestine of chickens. HS reduces the expression of LEAP-2 in the jejunum and the ileum of chickens and these effects could be mitigated by dietary GOS application.
The results in this chapter indicate that alterations in the expression of nutrient transporters play a role in the pathophysiology of HS, which when existing over a longer period is accompanied by a loss of body weight and is therefore of high economic relevance in poultry meat production. The results also emphasize the beneficial effects of GOS as an effective nutritional strategy that promotes the maintenance of the intestinal homeostasis under HS conditions.

Chapter 6 of this thesis was designed to investigate the effect of L-Arginine (L-Arg) supplementation on the intestinal epithelial integrity under HS conditions, using the Caco-2 model as presented in **Chapter 3**. We hypothesize that L-Arg, due to its function as a precursor in the synthesis of nitric oxide (NO), may be able to prevent HS-induced damage to the intestinal epithelial barrier. Caco-2 cells were pre-treated with non-toxic concentrations of L-Arg (0.4, 1, 4 mM) prior to exposure to HS (42°C). Results show that 4 mM L-Arg not only enhances the expression of HSP70 significantly, but also prevents the HS-induced reduction in nitric oxide (NO) levels, thereby preserving the intestinal integrity and preventing the downregulation and delocalization of E-cadherin. Inhibition of the inducible NO synthase (iNOS) markedly abrogates these beneficial effects of L-Arg, confirming that the beneficial effects of L-Arg supplementation are mainly attributable to its function as precursor in the NO synthesis. It can be concluded that L-Arg supplementation protects the intestinal epithelial integrity by maintaining NO synthesis and stabilizing E-cadherin expression under HS conditions.

In **Chapter 7**, we characterize the effects of the antioxidant α-lipoic acid (ALA) on HS-induced intestinal epithelial injury using the same Caco-2 cell model. ALA is recommended as food additive to reduce oxidative stress in a variety of clinical conditions and we hypothesized that it may successfully prevent epithelial cell damage under HS-conditions. After pre-treatment with ALA for 24h, Caco-2 cells were exposed to HS (42°C). Results indicate that ALA pre-treatment increases the HSP70 mRNA and protein expression, hampers the Nrf2 gene expression as well as the Nrf2 nuclear translocation under HS conditions, thereby stabilizing the cell

proliferation as demonstrated in the so-called wound healing assay.
It can also be concluded that the antioxidant ALA modulates not only the HS-induced oxidative stress response, but also prevents the disruption of intestinal barrier integrity by accelerating the reassembly of junctional complexes, preventing the delocalization of E-cadherin and stimulating the intestinal epithelial healing.

Main findings of this thesis

- Heat stress, as an environmental and occupational hazard, primarily affects the intestinal homeostasis by inducing damages to intestinal epithelium leading to loss of barrier integrity and an exaggerated inflammatory response.

- Induction of HSR is considered as a key adaptive mechanism in increasing the cellular thermotolerance to HS conditions and the modulation of HSR is an interesting target for intervention strategies to increase the resilience to HS.

- Differentiated Caco-2 cells grown on transwell inserts and mimicking the small intestine epithelial monolayer, can be considered as a suitable *in vitro* model to perform mechanistic investigations regarding the effect of HS on intestinal epithelial integrity and cellular adaptive responses.

- Broiler chickens are a suitable *in vivo* model to study the adverse effects of high environmental temperatures on gut health. Exposure to HS induces site-specific alterations in the intestinal integrity and homeostasis alongside the gastrointestinal tract.

- GOS can be an attractive dietary supplement for people/animals, who are at high risk to develop HS, since GOS can preserve intestinal integrity and reduce the inflammatory and oxidative reactions.

- L-Arg, the main precursor of cellular NO synthesis, promotes the HSR and maintains the intestinal integrity by preserving the NO levels in epithelial cells.

- ALA, as a well-known pro/antioxidant modulates not only the response to oxidative stress by increasing the HSR, but also successfully attenuates the disruption of intestinal integrity, accelerates the assembly of junctional complexes and supports cell proliferation and aids wound healing.

Annex

Nederlandse samenvatting

Wereldwijd verandert het klimaat onder invloed van uiteenlopende factoren. De afgelopen jaren is het duidelijk warmer geworden en er wordt voorspeld dat deze toename van de globale opwarming zal aanhouden in de toekomst. Hierdoor is er meer kans op hittestress, niet alleen door de stijging van de omgevingstemperatuur, maar ook door lichamelijke inspanning onder deze omstandigheden. Hittestress is gedefinieerd als een verstoring van de warmteregulatie in het lichaam waardoor de functie van verschillende organen beïnvloed wordt.
Bij hittestress verandert de mate van doorbloeding van de organen, waarbij de huid en perifere lichaamsdelen sterker doorbloed worden om voor afkoeling te zorgen. Dit heeft tot gevolg dat bijvoorbeeld het darmstelsel veel minder doorbloed wordt, waardoor een lokaal zuurstoftekort kan ontstaan en darmmotiliteit en het transport van nutriënten negatief beïnvloed wordt. De onvoldoende toevoer van zuurstof leidt dan tot oxidatieve stress op celniveau, beschadiging van eiwitten (verlies tertiaire structuur), een verstoring van de darmbarrière en uiteindelijk tot systemische ontstekingsprocessen.
Omdat een milde vorm van hittestress bij zware lichamelijke inspanning en ook bij koorts optreedt, beschikt het lichaam over een aantal fysiologische afweermechanismen, die de tolerantie van cellen tegen hoge temperaturen verhogen en de schadelijke effecten geïnduceerd door hittestress beperken. Deze interne afweermechanismen worden aangeduid als “hittestress response”, waarbij verschillende transcriptiefactoren en signaaltransductie trajecten betrokken zijn.
Een van de belangrijkste functionele elementen van dit afweersysteem tegen hittestress zijn specifieke “ stresseiwitten” (heat shock proteins, HSP). Ze functioneren onder andere als intracellulair chaperon bij aanmaak, stabilisatie en transport van eiwitten, controleren de afbraak van beschadigde eiwitten en regelen op deze manier de cellulaire eiwit homeostase.

In dit proefschrift wordt voornamelijk ingegaan op de kwetsbaarheid van het darmstelsel onder de condities van hittestress en de mechanismen die hierbij betrokken zijn. Op basis van dit inzicht in de cellulaire processen werden verschillende interventie- strategieën zowel *in vitro* als ook *in vivo* (in een model met kippen) getest om hun effectiviteit, met name betreffende hun beschermend effect op de darmbarrière te onderzoeken. De verschillende onderzoeken in het proefschrift kunnen in de volgende twee doelstellingen worden samengevat:

I) Het nader bepalen van cellulaire processen die betrokken zijn bij de “hittestress response”. Hiervoor worden *in vitro* modellen met darmepitheelcellen en een *in vivo* model met vleeskuikens gebruikt.

II) Het testen van verschillende nutritionele supplementen, die de schadelijke effecten van hittestress op de darmbarrière zouden kunnen beïnvloeden.

In **Hoofdstuk 2** van het proefschrift worden de effecten van hittestress op de darmbarrière besproken en worden de belangrijkste cellulaire

aanpassingsmechanismen, die betrokken zijn bij het ontwikkelen van tolerantie behandeld. Een van de meest voorkomende complicaties onder hittestress condities is het verstoren van de darmbarrière, waardoor antigenen, toxines en bacteriën de epitheliale cellaag kunnen passeren en lokale ontstekingsreacties in de darm, evenals een systemisch ontstekingsbeeld kunnen veroorzaken. Om dit te voorkomen, werden in het verleden verschillende nutritionele interventiestrategieën uitgetest. De doelstelling van een interventie is de schadelijke effecten veroorzaakt door hittestress, zoals hypoxia, verstoring van de darmbarrière en ontstekingen, op verschillende wijzen tegen te gaan. De verschillende mogelijkheden voor interventie worden eveneens in Hoofdstuk 2 nader toegelicht.

In **Hoofdstuk 3** wordt een *in vitro* systeem met darmepitheelcellen (Caco-2 cellen) beschreven dat geschikt bleek te zijn om de effecten van hyperthermie op de expressie van "stress eiwitten" en op de functie van de darmbarrière te onderzoeken. In dit model kon worden aangetoond dat de blootstelling aan cellen aan verhoogde temperaturen (40 en 42°C, 24 uur) de expressie van verschillende stress eiwitten (HSP70 en HSP90) en de expressie van haem oxygenase-1, een marker voor oxidatieve stress, duidelijk verhogen. Daarnaast kon blootstelling aan hittestress geassocieerd worden met een verstoring van de epitheliale weerstand en een verhoogde doorlaatbaarheid van de darmbarrière voor macromoleculen. De doorlaatbaarheid van de darmbarrière is afhankelijk van de integriteit van eiwitcomplexen, zoals tight en adherens junction eiwitten, die de epitheelcellen met elkaar verbinden, zodat ze een ondoorlaatbare cellaag vormen. Hoewel blootstelling aan hittestress geen effect had op de tight junction eiwitten van het darmepitheel, werd de expressie van de adherens junction, E-cadherin duidelijk beïnvloed door hittestress.
In dit systeem werd ook het mogelijk beschermende effect van niet-verteerbare oligosachariden (galacto-oligosachariden) op het handhaven van de integriteit van de darmbarrière tijdens hittestress onderzocht. Pre-incubatie van de cellen met galacto-oligosachariden verminderde inderdaad de hittestress response en had een positief effect op het behoud van integriteit van de darmbarrière door dislocatie van het adherens junction eiwit E-cadherin te voorkomen. Uit deze resultaten kan geconcludeerd worden dat galacto-oligosachariden een interessant voedingssupplement zijn voor individuen die een groot risico hebben om aan hittestress te worden blootgesteld.

In **Hoofdstuk 4** worden *in vivo* experimenten met vleeskuikens beschreven. In deze studie werden de effecten van hittestress op de homeostase in de darm nader onderzocht. De vleeskuikens werden gedurende 5 dagen voor zeker 8 uur per dag blootgesteld aan een omgevingstemperatuur van 38–39°C. Verschillende parameters gerelateerd aan de hittestress response en darmintegriteit, zoals de synthese van cytokines en hypoxia-gerelateerde markers werden gekozen om de

effecten van hittestress op de darmen van de vleeskuikens te bestuderen.
Deze studie toonde aan dat hittestress voornamelijk het jejunum en ileum in de darm beïnvloedt. De hittestress resulteerde in een verhoging van de expressie van stress eiwitten (HSP70 en HSP90), het adherens junction eiwit E-cadherin, de tight junction eiwitten claudin-5 en ZO-1, en de cytokines IL-6 en IL-8, in jejunum en ileum. De effecten in het jejunum veroorzaakt door blootstelling aan hittestress konden succesvol worden voorkomen in vleeskuikens, die een dieet kregen met galacto-oligosachariden, maar de hittestress effecten in het ileum werden door dit supplement niet significant verminderd. Deze resultaten laten voor het eerst bewijs zien dat er verschillen zijn in gevoeligheid voor hittestress in de verschillende delen van de darm. Tevens worden de resultaten van het voorgaand *in vitro* onderzoek, beschreven in Hoofdstuk 3, bevestigd want ook *in vivo* konden galacto-oligosachariden de darmintegriteit en het behouden van de homeostase in de darm bevorderen.

In **Hoofdstuk 5** worden aanvullende resultaten gepresenteerd met darmweefsel van het vleeskuiken experiment beschreven in Hoofdstuk 4. In dit aanvullende onderzoek werd de invloed van hittestress op nutriënten-transporters onderzocht, zoals de peptide transporter (PepT-1), de glutamaat/aspartaat aminozuur transporter-3 (EAAT-3), en de suiker transporters, fructose transporter (GLUT-5) en de natrium-afhankelijke glucose transporter 1 (SGLT-1). Het meest opvallende resultaat was de invloed (verhoging) van hittestress op de expressie PepT-1 en GLUT-5 in jejunum en ileum. Bij dieren, die het dieet met toevoeging van galacto-oligosachariden ontvangen hadden, kon deze reactie niet aangetoond worden en werd expressie van PepT-1 en GLUT-5 in het jejunum door hittestress niet verhoogd. Tevens werd in dit hoofdstuk het effect van hittestress op de expressie van de antimicrobiële peptide, LEAP-2, bepaald. Deze peptide is een belangrijk onderdeel van het aangeboren afweersysteem in de dunne darm van kippen. Hittestress verlaagt de expressie van LEAP-2 in het jejunum en ileum van de vleeskuikens en dit effect kan worden verminderd door galacto-oligosachariden toe te voegen aan het dieet.
De resultaten in dit hoofdstuk suggereren dat veranderingen in de expressie van nutriënten-transporters een rol spelen in de pathofysiologie van hittestress gerelateerde aandoeningen. Indien veranderingen in de expressie van nutriënten-transporters lang aanhouden, zal dit samengaan met gewichtsverlies en daarom is het reguleren van de expressie van deze transporters een relevante doelstelling voor vleeskuikenhouders. Tevens benadrukken de resultaten de positieve effecten van galacto-oligosachariden als een waarschijnlijk zeer effectieve nutritionele strategie om de handhaving van de homeostase in de darm tijdens hittestress te bevorderen.

Hoofdstuk 6 van het proefschrift was opgezet om het effect van het aminozuur, L-Arginine (L-Arg), op de integriteit van de darm tijdens hittestress te onderzoeken.

Hierbij werd gebruik gemaakt van hetzelfde *in vitro* darmepitheel model zoals beschreven in Hoofdstuk 3. De hypothese was dat L-Arg, vanwege zijn functie als precursor in de synthese van stikstofmonoxide (NO), mogelijkerwijs kan bijdragen aan het voorkomen van verstoring van de darmepitheelbarrière veroorzaakt door hittestress. In deze onderzoeken werden de darmepitheelcellen behandeld met niet-toxische concentraties van L-Arg (0.4, 1, 4 mM) voorafgaand aan de blootstelling aan hittestress (42°C). Resultaten laten zien dat 4 mM L-Arg niet alleen de expressie van het stress eiwit HSP70 verhoogt, maar ook de verstoring van de stikstofmonoxideproductie door hittestress kan voorkomen. Hierdoor wordt ook een verlaging van de expressie van E-cadherin voorkomen en de integriteit van de darmbarrière beter behouden.
Aanvullend onderzoek kon bevestigen dat de effecten van L-Arg voornamelijk toe te schrijven zijn aan zijn functie als precursor in de synthese van stikstofmonoxide, want na remming van het enzym iNOS, dat bijdraagt aan de synthese van het stikstofmonoxide, konden de positieve effecten van L-Arg niet meer worden gezien.
Uit de resultaten van dit hoofdstuk kan worden geconcludeerd dat toevoeging van L-Arg, de integriteit van het darmepitheel beschermt door het handhaven van de synthese van stikstofmonoxide en het stabiliseren van de E-cadherin expressie tijdens hittestress.

In **Hoofdstuk 7** worden de effecten van het antioxidant, α-lipoic acid (ALA), op de verstoring van de darmepitheelbarrière veroorzaakt door hittestress onderzocht in het *in vitro* darmepitheel model. ALA wordt reeds aanbevolen als voedingssupplement om cellulaire oxidatieve stress te verlagen in klinische omstandigheden waarbij oxidatieve stress een grote rol speelt. Daarom werd ervan uitgegaan dat dit antioxidant waarschijnlijk de beschadiging van de darmepitheelcellen tijdens hittestress kan voorkomen.
In het *in vitro*-model werden de cellen 24 uur geïncubeerd in aanwezigheid van verschillende concentraties van ALA, alvorens te worden blootgesteld aan hittestress (42°C). De resultaten tonen aan dat behandeling met ALA de expressie van het stress eiwit HSP70 verhoogt, terwijl de expressie van het Nrf2 gen (transcriptie factor, die de expressie van antioxidant eiwitten reguleert) verminderd is tijdens hittestress. Tevens stabiliseert ALA de proliferatie van epitheelcellen zoals aangetoond in de zogenaamde wondheling assay.
Uit de resultaten kan worden geconcludeerd dat het antioxidant ALA niet alleen de oxidatieve stress response geïnduceerd door heat stress kan verminderen, maar dat ALA ook de verstoring van de integriteit van darmepitheelbarrière kan voorkomen door de de-lokalisering van E-cadherin te tegen te gaan en de proliferatie van het darmepitheelcellen te stimuleren.

De belangrijkste bevindingen in dit proefschrift:

- Hittestress is een toenemend gezondheidsrisico, dat voornamelijk de homeostase in de darm beïnvloedt door schade aan te brengen aan het darmepitheel. Dit kan leiden tot verstoring van de integriteit van de darmbarrière en het ontstaan van uitgebreide ontstekingsreacties.

- Inductie van de zogeheten hittestress response wordt beschouwd als een van de meest belangrijke mechanismen, dat bijdraagt aan tolerantie ontwikkeling tegen hittestress. Modulatie van deze hittestress response is de basis voor het ontwikkelen van interventiestrategieën, die mogelijk de tolerantie tegen hittestress kunnen verhogen.

- Het *in vitro* model, waarin gedifferentieerde darmepitheelcellen (Caco-2 cellen) controleerbaar een impermeabele monolaag op het membraan van een insert in celkweekplaten vormt, kan worden beschouwd als een geschikt model voor mechanistische studies, die het effect van hittestress op de integriteit van het darmepitheel en de daarop volgende immuunreactie bestuderen.

- Vleeskuikens zijn een geschikt *in vivo* model om de schadelijke effecten van hoge omgevingstemperaturen op de darmgezondheid te bestuderen. Blootstelling aan hittestress veroorzaakt specifieke veranderingen in de integriteit en homeostase aan de verschillende onderdelen van het darmstelsel.

- Galacto-oligosachariden zijn een aantrekkelijk voedingssupplement voor mens en dier, die een verhoogd risico hebben om hittestress te ontwikkelen. Dit omdat galacto-oligosachariden de darmintegriteit bevorderen en de cellulaire oxidatieve stress en de ontstekingsreacties verminderen.

- Het aminozuur L-Arginine, de belangrijkste precursor voor de synthese van stikstofmonoxide (NO), stimuleert de beschermende hittestress response door behoud van stikstofmonoxidebalans in de darmepitheelcellen en de integriteit van de darmepitheelbarrière.

- Het vetzuur α-lipoic acid (ALA), reeds bekend als een antioxidant, moduleert niet alleen de oxidatieve stress reactie door het stimuleren van de hittestress response, maar vermindert tevens succesvol de verstoring van de integriteit van het darmepitheel, bevordert de wederopbouw van eiwitcomplexen die voor epitheelintegriteit zorgen (bv. E-cadherin), en ondersteunt de proliferatie van epitheelcellen, zoals aangetoond in de wondheling assay.

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A

Curriculum Vitae

Soheil Varasteh was born in Urmia, Iran on July 2, 1986. After finishing high school and taking part in the National University Entrance Exam, he was accepted to study Veterinary medicine in Urmia University located in his home city. During this study, he was appointed as the member of Young Elite Society of Iran because of his efforts in designing and patenting a novel device for taking samples from laboratory animals. He successfully obtained his Doctor of Veterinary Medicine degree in December 2011. His interest in basic sciences, in particular Pharmacology, led to his decision to take part in the Drug Innovation PhD program of the Graduate School of Life Sciences in Utrecht, the Netherlands. His PhD project at Utrecht University was a joint project between the Utrecht Institute of Pharmaceutical Sciences (UIPS), division Pharmacology and the Institute for Risk Assessment Sciences (IRAS), under the supervision of Prof. dr. J. Garssen, Prof. dr. J. Fink-Gremmels, Prof. dr. A.D. Kraneveld and Dr. S. Braber. His research aimed to expand the current knowledge about the vulnerability of intestinal barrier to heat exposure. Additionally, his research aimed to introduce pharmaco-nutritional approaches to mitigate the adverse effect of heat stress in the gastrointestinal tract. The results from this research are presented in this thesis.

List of Publications

Varasteh S, Fink-Gremmels J, Garssen J, Braber S. α-lipoic acid ameliorates the intestinal epithelial monolayer damage under heat stress conditions: model experiments in Caco-2 cells. *Submitted for publication*

Varasteh S, Braber S, Garssen J, Fink-Gremmels J. Galacto-oligosaccharides exert a protective effect against heat stress in a Caco-2 cell model. J Funct Foods. 2015;16: 265–277. doi:10.1016/j.jff.2015.04.045

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