The work of this thesis was carried out in close cooperation with Nutricia Research and FrieslandCampina and was part of the CCC WP-25 program: Immunomodulating properties of oligosaccharides from various sources.
The author gratefully acknowledges the generous support of the Nutricia Research.

Printing of this thesis was financially supported by: Nutricia Research and Utrecht Institute for Pharmaceutical Sciences (UIPS).

Cover photo: Sona Moayedzadeh (was taken at Urmia, Iran).

Cover design and Layout: Arash Alizadeh

Printed by: Gildeprint, Enschede, The Netherlands

ISBN: 978-94-6233-381-9

The challenge of early life exposure to deoxynivalenol: Intestinal vulnerability and intervention strategies

De uitdaging van een blootstelling aan deoxynivalenol in de eerste levensfase: de kwetsbaarheid van de darm en mogelijkheden voor bescherming

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G. J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
woensdag 12 oktober 2016 des middags te 2.30 uur

door

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All men who have achieved great things
have been great dreamers

Orison Swett Marden

To My Family

Contents

Chapter 1

General introduction

General introduction

Humans and animals are frequently challenged by mycotoxins which are among the most prevalent natural contaminants in food and feed. Mycotoxins are low-molecular weight, secondary fungal metabolites with diverse chemical and toxicological properties [1–4]. In general, the main toxic effects induced by mycotoxins include nephrotoxicity, hepatotoxicity, genotoxicity, myelotoxicity, teratogenicity, potential carcinogenicity and adverse effects on the reproductive system as well as immunomodulatory effects [1–3]. Assessing the impact of mycotoxins on the human population, infants are recognized as a potentially vulnerable subgroup. Infants have a higher consumption of milk and/or food per kg body weight, a higher metabolic rate, but incompletely developed detoxification mechanisms compared to adults [5–7]. The trichothecene deoxynivalenol (DON) has received particular attention as the rate of contamination of cereal grains, the staple food in many parts of the world, seems to increase rapidly [8–11]. This high exposure rate to DON was confirmed by urine analysis in adults and children [12–14].
DON is a small and recalcitrant molecule, resisting common food processing [15,16]. The importance of DON is not limited to the parent molecule, since a number of studies refer to related fungal (3- and 15-acetyl DON) metabolites, as well as the plant metabolite (DON-3-glucoside), which may contribute to the overall body burden associated with the consumption of contaminated grains [17–22].
The intestinal tract as the largest interface between host and environment is of major importance for nutrient utilization, health and well-being. The gastrointestinal tract has a unique structure and architecture, contains a very large surface area (about 400 m^2 in humans) and harbours a diverse microbial community and complex mucosal immune system [23–26]. The gastrointestinal tract serves as an important organ, which plays a pivotal role in host and environment interactions and the intestinal epithelium constitutes an effective barrier against intraluminal allergens, pathogens and toxins.
The intestinal epithelial barrier is one of the first targets for food contaminants, including mycotoxins. Indeed many investigations, both *in vitro* and *in vivo*, confirm that especially the mycotoxin DON acts as a trigger for intestinal integrity breakdown [27–30]. In addition, other trichothecene mycotoxins, as well as fumonisins, ochratoxin, patulin and even aflatoxins can also impair the intestinal barrier integrity [31–35]. Impairment of the intestinal barrier integrity is accompanied by an increased paracellular flux of antigens and even pathogens and a persistent inflammatory reaction [27], which can lead to chronic inflammatory reactions, allergies and auto-immune diseases [36–39]. The relation between DON exposure and the risk for allergic reactions was recently demonstrated, when model experiments in mice showed that DON facilitates allergic sensitization to the food protein whey [40].

The pig is one of the most sensitive animal species regarding DON exposure. This is not only of importance for the animal health sector, but is also an indication of the

vulnerability of the human population. The anatomical and physiological similarities in the intestinal tract of pigs and humans are well recognized [41–43] and different studies demonstrated the resemblance between piglets and human infants with regards to the postnatal gut development and nutritional requirements [43–45]. Therefore, new-born piglets provide a valuable animal model for studying the development of gastrointestinal tract, adaptation and metabolism of nutrients, parenteral nutrition, various dietary ingredients and for surgical purposes [46–50]. Hence, in the current thesis, we selected the piglet model to investigate the effect of exposure to common dietary contamination levels of DON as well as to assess the impact of dietary galacto-oligosaccharides (GOS) on morphological and functional parameters related to gut health.

Non-digestible oligosaccharides, including GOS and fructo-oligosaccharides (FOS), have been recognized as valuable additives for infant formulas, resembling the various human milk oligosaccharides present in breast milk. Different clinical investigations demonstrated a comparable intestinal microbial composition between breastfed infants and infants given a formula supplemented with GOS/FOS [51,52]. These oligosaccharides are assumed to act as prebiotic substances supporting the development of a healthy intestinal microbiota and the associated immune system [53–56].
An increasing body of evidence indicates that early colonization of the intestinal tract also affects the immune reactivity in later phases of life [57]. Different studies performed by our group and others demonstrated that the beneficial effects of GOS are not limited to microbiota-dependent mechanism, since GOS could also exert direct effects on the intestinal epithelial barrier and corresponding inflammatory reactions in different *in vitro* models in the absence of micro-organisms [58–60].

Aims and outlines of the thesis

Worldwide prevalence of the food and feed contaminant DON is recently rising, which is particularly due to the global climate change. Although different strategies have been applied to control the DON problem at pre- and postharvest steps, human (especially children) and animal exposure to DON is still poorly controlled. The first part of the thesis aims to gain closer understanding of the adverse effects induced by DON on intestinal barrier and gut health in a well-defined intestinal epithelial cell (Caco-2) model as well as in a piglet model. Moreover, GOS were investigated as possible intervention strategy against this DON challenge in piglets. The second part of the thesis aims to describe the gut health-promoting effects of GOS in an introduced neonatal piglet model. In addition, the direct interaction between intestinal epithelium and GOS was investigated in a long-term *in vitro* exposure model aiming to get more insight into GOS effects on intestinal epithelium. The possible mechanism of action for the direct, microbiota-independent, effects of GOS on intestinal epithelium was examined in a preliminary study described in the last part of the thesis.

The following main objectives were addressed in this thesis:

Chapter 1: General introduction, characterizing the aims and outline of the thesis.

Chapter 2: In recognition of the increasing concerns related to the frequent occurrence of mycotoxins in food and animal diets, the available literature related to possible intervention strategies to prevent exposure by means of sequestering agents or functional food and feed additives that might be able to mitigate specific adverse effects of mycotoxins were summarized in a brief review. Special attention was given here to DON and related trichothecenes.

Chapter 3: In this chapter the differences between DON and its fungal, plant and bacterial metabolites regarding their adverse effects on intestinal epithelial cells (Caco-2) is discussed. Also the capability of GOS to attenuate these adverse effects of DON and its derivatives were investigated in this chapter.

Chapter 4: The investigations described in this chapter demonstrate that low-level DON exposure, even after a short exposure period can impair the growth performance of piglets and induces distinct changes in the intestinal tract. These data should contribute to a closer understanding of the DON-associated impairment of gut health, and illustrate in a detailed approach the effects of DON on the individual segments of the intestinal tract.

Chapter 5: Here we present the outcome of an *in vivo* intervention study in which GOS were given to piglets exposed to a DON-contaminated diet. From this chapter it is concluded that GOS can be promising candidates to mitigate the DON-induced adverse effects on the intestinal architecture of piglets.

Chapter 6: This chapter was designed to validate a neonatal piglet model for paediatric research. At the same time, the effect of dietary GOS was studied in early life. It can be concluded that GOS stimulate the development of the intestinal microbiota, improve the intestinal architecture and seem to modulate barrier integrity and parameters of the innate immune system in healthy neonatal piglets.

Chapter 7: Considering that GOS were already known to enforce tight junction assembly, the aim of this chapter was to study the effect of GOS on non-differentiated Caco-2 cells and follow its effects until the stage of fully differentiated cells, resembling the intestinal barrier.

Chapter 8: This chapter provides preliminary results for GOS effects on mitogen-activated protein kinases signalling pathways aiming to unravel the possible mechanism

of action for microbiota-independent effects of GOS.

Chapter 9: In a comprehensive discussion, the results obtained in the current investigations are discussed in terms of the underlying mechanisms as well as their overall relevance and clinical applicability. Finally, future research directions are indicated.

Chapter 10: The comprehensive summary of the most relevant findings of the current thesis is presented.

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Chapter 2

Mitigating the adverse effects of mycotoxins: opportunities and limitations (a brief review)

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Introduction

Mycotoxins are small and stable molecules, produced as secondary metabolites of fungal species. Particularly, the genera of *Aspergillus*, *Penicillium* and *Fusarium*, *Alternaria* and *Claviceps* are known as toxigenic moulds invading crops at the pre- and post-harvest stage. Mycotoxins are currently considered as the most frequently occurring undesirable contaminants in agricultural commodities on a global level [1–4]. Their prevalence in food and feed is still increasing, and changes in agricultural practice, the increased use of fungicides and pesticides as well as the changing global climate, have been discussed as possible causes of such an increasing occurrence in the last decades [3,5]. While cereal grains and corn (maize) are of major concerns as staple foods, high mycotoxin concentrations may also be found in oil seeds, nuts, fruit and fruit juices, grapes and wines as well as in spices. Despite their diverse chemical structure, almost all mycotoxins are stable under the conditions of food and feed processing [6,7].

Initially, mycotoxin producing fungi and their mycotoxins have been allocated to two subgroups based on the time of invasion of the plant.

The first group is soil-borne and invades the plant at the pre-harvest stage. This results not only in the pre-harvest contamination of cereals and grains, but also in plant-fungal interactions, which is extensively described for *Fusarium* species [8–10]. Fusaria (and other fungal species) excrete mycotoxins into their neighbouring environment, and in turn, plant cells aim to detoxify such toxins by conjugation to glucose or sulphate moieties. These so-called modified mycotoxins (previously also called masked mycotoxins) remained undetected for a long time, as conjugation changes the physico-chemical properties significantly, and hence they were not extracted by commonly used solvent extractions. Such modified forms have been described for deoxynivalenol (DON), T-2 toxin, zearalenone (ZEA) and fumonisin and new conjugates still emerge with the increasing awareness of the potential presence of such conjugates. Upon ingestion with contaminated foods and feed, microbiota in the intestines can easily cleave the conjugates, and the liberated parent toxin, contributes to the overall exposure of humans and animals [11].

Fusarium species are the main producers of the important class of trichothecenes, comprising more than 180 structurally related compounds, as well as the group of fumonisins with their unique sphingolipid structure, ZEA, the only mycotoxin with hormonal activity, and the enniatin group exhibiting ionophoric activity. Other typical pre-harvest mycotoxins, are the complex group of ergot alkaloids as well as indole derivatives, produced by *Epichloë* species, with the common families of *Claviceps* and *Neotyphodium* species [1,3].

Next to these pre-harvest fungi invading living plants at different stages of propagation, the second group, composed of *Aspergillus*, *Penicillium* and *Alternaria* species were previously classified as post-harvest moulds, found predominantly on stored grains, nuts, fruit or other plant-derived commodities. The strict distinction between pre- and

post-harvest moulds has been revisited in the light of many findings indicating that the above mentioned fungal species are also found in the living plant. This applies for example to Aspergilli, which seem to adapt to modern agricultural techniques rapidly and infect the living plant, such as soya beans and the corn cobs or nut trees. The observation that these moulds continue and even increase their toxin production at the post-harvest stage still discriminate them from the pre-harvest fungal species with no or limited mycotoxin production during storage. The knowledge about the major time point of toxin production, either at the pre-harvest or post-harvest stage, is also used as a guidance for targeted intervention strategies [7].

Significant economic losses are associated with the increasing prevalence of fungal species. Recent estimates of the EU (EXPO 2015) consider total economic losses of more than 10% in crop production at the pre-harvest stage already [12,13]. In addition, and even more importantly, the increasing prevalence of mycotoxin contamination comprises a significant risk to human and animal health [14]. In turn, statutory limits (maximum levels) in food and feed materials have been set by WHO, JECFA, the American FDA, the European EFSA, and by numerous countries in individual assessments. The implementation of such statutory limits, however, varied and largely depended on the capacity of individual countries to set up competent analytical control laboratories for the analysis of large numbers of samples. Furthermore, numerous attempts were made to reduce the toxin burden of agricultural commodities. These include improvement of plant cultivation techniques, selection for resistant plant varieties, including genetically modified plants, such as maize, carrying the *Bacillus thuringiensis* (Bt) gene conveying repellent activity to insects and thereby preventing invasion by fungal species. Moreover, targeted controls of grains and cereals, based on climate recordings identifying high risk regions, are implemented.

Toxicity and impact of mycotoxins on human and animal health

Mycotoxins originate from different biosynthetic pathways in filamentous fungi and may be clustered into chemically related toxin groups, such as the aflatoxins (aflatoxin B_1 (AFB_1), AFB_2, AFG_1, AFG_2, sterigmatocystin) [15], the groups of fumonisins (with fumonisin B_1 (FB_1), FB_2 and FB_3 as major representatives) [16], the ochratoxins (ochratoxin A (OTA), OTB and OTC) [17] and the complex group of trichothecenes [18]. No structural similarities exist across these groups hence the mechanism of toxicity, the target organs and the overall toxicological effects are highly diverse.

It is beyond the scope of this review to describe the toxicity of individual mycotoxins in detail. With regards to human health, interest still focusses on the potential carcinogenic mycotoxins, such as aflatoxins (associated with human hepatic cell carcinomas and hepatotoxicity in all animal species tested), ochratoxin A (OTA), associated with renal diseases and probable the prevalence of urinary tract tumours as well as renal cell carcinoma in rodents, and FB_1, primarily associated with oesophageal cancer and recently gaining attention as inducer of neuronal tube defects in rodents (for review

see ref. [3]). More recently also non-cancer endpoints are considered, including the interaction of mycotoxins with infectious agents and overall health status [19]. These findings, as well as the effect of mycotoxins on the intestinal barrier facilitating the translocation of pathogens and allergens might be implicated in a variety of human chronic diseases, such as food allergies and inflammatory bowel disease [20]. Another emerging concern is the exposure of humans and animals to often complex mixtures of mycotoxins, depending on their diet. Taken together, the findings of the very frequent and currently unavoidable exposure of human and animals have resulted in numerous approaches to prevent, inactivate or reduce the toxic effects of mycotoxins. The main principles of the individual approaches will be summarized in this review.

Attempts to mitigate the adverse effects of mycotoxins with chemical or microbiological agents

The first approach to minimize the impact of mycotoxins on human and animal health was directed to the aflatoxins, based on the early recognition of their carcinogenicity for humans, and their severe impact on animal health and productivity, particularly regarding poultry. With the aim to allow the safe consumption of aflatoxin-containing basic foods, such as corn (maize) and peanuts, sorting and dehulling of corn, soya and nuts was recommended. These procedures are time consuming and can only be done for small batches. Given the typical fluorescence of aflatoxins (blue for AFB_1 and AFB_2, green for AFG_1 and AFG_2), nuts and figs are often also sorted semi-automatically under UV light. In Asia, traditional fermentation techniques using non-toxigenic fungi (like *Aspergillus oryzae*) have been widely applied in the past. More recently, Lactobacilli and other bacteria and fungi and/or their enzymes are tested with the aim to identify possible new mitigating procedures that can be applied rapidly in humans or animals.

The first functional food additive developed as protective agent against aflatoxin-contaminated foods was a potassium-sodium silica-clay, which can bind aflatoxin in the presence of water and hence will sequester AFB_1 in the intestinal tract thereby preventing its absorption [21]. This product (Novasil™) received marketing authorization in different countries and its use was also advised by the WHO in clinical cases of human aflatoxicosis. Based on these initial findings, subsequently, numerous related silica-clay products have been developed, particularly as feed additives to reduce the bioavailability and hence toxicity of mycotoxin contaminated feed (for review see also [22–24]).

In the 1990s, also chemoprotective (anti-cancer) agents, such as oltipraz (5–(2-pyrazinyl)–4–methyl-1, 2-dithiole-3-thione), were developed for applications in humans with a high risk of aflatoxin exposure [25]. Oltipraz is a modifier of mammalian hepatic enzymes, inhibiting CYP450 enzymes and hence reducing the bio-activation of aflatoxin into its epoxides, while at the same time increasing the expression of phase II biotransformation pathways, resulting in a more rapid detoxification of aflatoxin and the anti-oxidant capacity of (liver) cells [26]. Finally, vaccination programs against hepatitis

B were proposed in consideration of the common mutational hotspot of hepatitis B virus and aflatoxin on mammalian liver cells to reduce the risk for liver cancer [27]. None of these strategies was widely implemented as the risk-benefit analysis of these intervention strategies failed to show significant benefits.

While initially, all efforts focussed particular on the aflatoxins in foods for human consumption, the increasing prevalence of mycotoxins in animal feeds resulted in numerous activities to identify substances with a broad protective effect against different mycotoxins, not only aflatoxins [28,29]. Such feed additives to be used in animal diets require marketing authorization within the EU (EU 18881/2003) (to which as special category for mycotoxin mitigating agents was added), but only very few products have been licensed as yet. According to the criteria defined in this EU regulation, two major classes of compounds can be considered, the so-called mycotoxin-adsorbents that aim to reduce the bioavailability of mycotoxins and promote their (unchanged) excretion, and the biotransforming agents, that aim to (enzymatically) modify mycotoxins, rendering less toxic or non-toxic degradation products [22].

1) Inorganic mycotoxin adsorbents

Mycotoxin adsorbing agents (also denoted entero-adsorbents, as their main mechanism of action is to sequester mycotoxins in the intestinal tract), are generally polymers with a large absorption surface. The adsorption (binding) process may involve different intermolecular interactions, such as hydrogen binding, van der Waals forces and electrostatic attraction, which depend on the chemical structure of the mycotoxin and the adsorbent [30,31]. The efficacy is generally tested in simple buffer systems under different pH conditions [32,33]. The experimental protocols are variable and may include single concentration studies, isotherm studies (fixed binder concentration and increasing concentrations of the toxin), as well as complex models of the gastrointestinal tract, comprising digestive enzymes as well as artificial digesta [34–36], ideally followed by verification experiments in target animal species [37]. Ultimately, the (theoretical) number of binding sites in polymers used as binding agents as well as the lipophilic or hydrophilic balance under the different pH conditions in the stomach and the intestines of animals, determine the rate of absorption following the ingestion of contaminated foods.

Activated charcoal, cholestyramine and polyvinylpyrrolidone: the reference adsorbents

A mycotoxin adsorbent agent should not be absorbed from the intestines and must scavenge mycotoxins already in the stomach and in the upper intestine prior to absorption. For testing the adsorptive capacity, **activated charcoal** [38,39] has been widely used as a reference compound, in recognition of its well-known medical use as detoxifying agent.

Cholestyramine is an insoluble quaternary ammonium anion exchange resin, which has

a strong affinity for binding to anionic compounds [40–42]. It is used in conventional medicine to bind bile acids in the gastrointestinal tract and reduce plasma concentrations of low-density lipoprotein (LDL) and cholesterol [39]. In different *in vitro* studies, the binding capacity of cholestyramine has been reported for OTA [41], FB_1 [42] and ZEA [43,44]. **Polyvinylpyrrolidone (PVP)** is a cross-linked synthetic polymer, which is able to make a hydrogen hull around its molecules and to adsorb polar molecules, such as aflatoxins [43,45].

Although these substances have been used in technical processes and as reference materials in adsorbent studies, they have a very limited use as feed additives, as the use of higher concentrations, and any long term use, will result in nutritional imbalances. The investigations with activated charcoal pointed, however, to a number of factors affecting the adsorption properties, such as surface area and pore size distribution [46,47]. Moreover, the polarity and hydrophobicity of individual mycotoxins, correlated with the binding efficiency indicated for example by high percentage scavenging of ZEA, but a rather low binding efficiency for DON and nivalenol [38].

Silica clays

Hydrated sodium calcium aluminosilicate (HSCAS) was first reported to adsorb AFB_1 with high affinity and capacity in *in vitro* systems [21,48–52]. This binding efficacy is attributed to an electron donor-acceptor (EDA) mechanism, where the negative surface of clay attracts the two polar β-dicarbonyl esters of the aflatoxin molecule [53]. This mechanism explains also that HSCAS adsorbs particularly AFB_1, but not less polar molecules, such as DON or ZEA.

HSCAS has a very large surface area. The isotherm shape for AFB_1 sorption onto HSCAS is biphasic showing that the maximum amount of AFB_1 adsorption by HSCAS is 0.336 mol/kg (72.9% of maximum capacity in theory) [49]. These findings suggest that the binding of AFB_1 to HSCAS mainly take place within the interlayer region of the clay.

While HSCAS was the first clay identified to scavenge aflatoxin in a watery solution (including the stomach and the intestines), subsequently other crystalline hydrated aluminosilicate molecules were investigated, such as montmorillonite, smectite, illite, kaolinite, biotite and clinoptilolite and other phyllosillicates [54]. Crystallography allows the classification of phyllosilicates based on their tetrahedral (commonly Si^{4+}, Al^{3+} and Fe^{3+}) and octahedral (commonly Al^{3+}, Fe^{3+}, Mg^{2+} and Fe^{2+}) structures.

Hydrogen bonds are crucial for the adsorption property of aluminosilicates, but the number of OH groups is pH dependent [53]. In turn, recent attempts focus on an increase of the cation exchange mechanism via coatings resulting in a higher protonation at a low pH to force adsorption already in the (acidic) stomach [55].

Bentonite, originating from volcano ash, has been widely used as feed additive as anti-caking agent, and recently as mycotoxin sequestering agent. Bentonite contains mainly montmorillonite, a hydrated silicate sodium-aluminium-magnesium hydroxide. The adsorption properties of bentonites vary due to the presence of different cations (Na^+,

K^+, Ca^{2+}, and Mg^{2+}) in the layers [24,56]. Different bentonite based adsorbents show a binding capacity of > 95% for AFB_1. This high binding capacity is obtained independent from the pH of the buffer system [32].

Zeolites are crystalline hydrated tektoalumosilicates consisting of three-dimensional frameworks of SiO_4^{4-} and AlO_4^{5-} which are linked through shared oxygen atoms [57]. This structure allows the formation of internal surfaces (channel-like) increasing the overall surface to about 1000 m^2 per g of zeolite.

Currently, a number of modified zeolites has been developed, considering that modification of the negatively charged minerals and adjustments in hydrophobicity might improve the binding capacity to less- or even non-polar mycotoxins. These modified zeolites (organo-zeolites) contain different amounts of octadecyl dimethyl benzyl ammonium chloride and dioctadecyl dimethyl ammonium chloride obtained by wet and dry processes. They bind not only to AFB_1, OTA, and ZEA, but also to various ergopeptide alkaloids, but not to the trichothecene DON [58,59]. Extending the binding capacity of zeolites and other mineral clays increase, however, the risk that also important nutrients are scavenged [60]. In general, there is a number of concerns related to the broad use of inorganic adsorbents, such as their limited adsorption capacity for many mycotoxins (others than aflatoxins), the potential environmental accumulation after excretion [29,61], and the already mentioned non-specific binding of minerals and vitamins in body [62]. Such concerns are addressed in a risk-benefit analysis, as part of the licensing process.

2) Organic polymers

Micronized fibers

Dietary fibers are defined as the edible part of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine [63–65]. Dietary fibers can be classified based on their solubility in water: the soluble fibers are pectins, β-glucans, glucomannans, fructooligosaccharides (FOS) and inulin, whereas water-insoluble fibers consist of cell wall components, such as cellulose, hemicellulose lignin and hemicelluloses. Consumption of fibers with grain products and vegetables is recommended to improve digestion, intestinal passage rate and stool consistency. Dietary fibers may also reduce the absorption of undesirable toxins from the intestinal lumen and have been recommended as a non-specific preventive measure [65]. For example, the effectiveness of micronized wheat fibers for adsorption of OTA has been reported in rats and piglets [66,67], but in general, more targeted approaches, such as the use of yeast cell wall or bacterial cell wall seem to be more effective.

Yeast and yeast cell wall products

The yeast cell (for example of *Saccharomyces cervisiae*) contains large amounts of polysaccharides, such as mannans and glucans (β1, 3-glucan), and display adsorption properties with multiple mechanisms for binding, like hydrogen bonding

and ionic or hydrophobic interactions [68,69]. Chemically, mannans are highly branched carbohydrates bridged by phosphodiester bridges and forming together with the polypeptide backbone a relatively rigid cell wall [69]. A number of studies has addressed to yeast cell wall as organic compounds with the capability of forming complexes with mycotoxins [69–71]. Again, polarity, solubility, size, specific shape and charge distribution and dissociation constants for individual mycotoxins and individual yeast cell wall products determine the ultimate adsorption quality [72]. For example, Yiannikouris *et al.* [69] described the correlation between the amount of β-D-glucan in yeast cell and the ability to bind ZEA non-covalently. If the yeast cell wall contains higher levels of chitin, the binding capacity for ZEA is decreased. Moreover in a comprehensive study utilizing different mycotoxins and β-D-glucans over a pH range (3.0, 6.0 and 8.0), a higher affinity for mycotoxins such AFB_1 > DON > OTA was observed at low pH values, whereas alkaline conditions were favourable for the adsorption of patulin [70]. Yeast cell wall products are currently widely used as feed additives, and exhibit prebiotic activity in addition to their direct mycotoxin scavenging capacity, which supports the mitigation of adverse effects of mycotoxins on the intestines and the associated immune system.

Bacteria, including lactic acid bacteria

Lactic acid bacteria are belong to of the most important and widely used microorganisms in fermented foods and form an important (desirable) fraction in intestinal microflora [73]. The cell wall of lactic acid bacteria consists mainly of peptidoglycans and polysaccharides and these components provide the binding sites (hydrophobic pockets) for diverse mycotoxins non-covalently [74,75]. In turn, it has been demonstrated that for example both viable and heat- or acid-killed *Lactobacillus rhamnosus* strains are able to effectively remove the AFB_1 from aqueous solutions and this effect was pH independent [75,76]. In a study by El-Nezami *et al.* [77] it was reported that all the tested *Lactobacillus rhamnosus* strains were more effective than *E.coli.* Lactic acid bacteria have been also specifically tested for their binding capacity for AFM_1, the aflatoxin metabolite excreted into milk. Overall, the binding affinity was less than that observed for AFB_1, probably due to the higher polarity of AFM_1 [75,78,79].

Comparable findings were reported for the binding of trichothecenes, such as DON, diacetoxyscirpenol (DAS), nivalenol (NIV) and fusarenon-X (FX) by lactic acid bacteria, again described as non-covalent adherence with no modification of the molecular structure. Hence, it is concluded that the specific polysaccharide and peptidoglycan components of bacterial cell wall determine the variability in the binding efficacy of individual bacterial strains. Heat treatment might even increase the binding of trichothecenes and AFB_1 to constituents of bacterial cell wall, which are not available in the intact bacterial cell wall [80,81]. Moreover, it is speculated that a breakdown in glycosidic linkages of polysaccharides as well as a cleavage of the amide linkage in peptides and proteins under acidic conditions result in an increased pore size leading to the improved binding of mycotoxins, like AFB_1 and ZEA [81,82]. The binding efficacy

of Lactobacilli (particularly the LGG and LC-705 strains) is also reported for ZEA as well as OTA [82,83]. Moreover it has been reported that both FB_1 and FB_2 are effectively removed by different strains of lactic acid bacteria, but it should be stressed that considerable differences were detected between different strains and the bacteria were less efficient against FB_1 [84]. Here, the tricarballylic acid (TCA) chain in the fumonisin molecule plays a role in binding, since the hydrolysed fumonisin had less binding affinity to lactic acid bacteria [85].

3) Mycotoxin biotransforming organisms and enzymes

During ingestion (or in some cases prior to feeding in fermentation systems) mycotoxin biotransforming agents intend to modify the chemical nature of the mycotoxin rendering a degradation product that is less toxic. As it has been recognized that bacteria and fungi can enzymatically degrade and deactivate most mycotoxins by ring cleavage, hydrolysis, de-epoxydation, deamination and decarboxylation [86], research focusses on the identification of suitable probiotic strains and the isolation of enzymes from diverse microorganisms. The source of mycotoxin-degrading fungi and bacteria is generally the soil and plant material, but also the digestive tract of animals, particularly the rumen, as the rumen microbiota are effectively modify mycotoxins [86].

Cleavage of the cyclopentanone ring of AFB_1

Reduction of the C-3 keto group in the cyclopentanone ring of AFB_1 results in the formation of (non-fluorescent) aflatoxicol, the common metabolite of the rumen microbiota as well as other microorganisms, such as for example *Cornybacterium rubrum*. *Rhizopus* species and *Hypomyces rosellus* [86–88]. As this first reduction step, reduces certainly mutagenicity (DNA adduct formation), hepatotoxicity is incompletely reduced and the risk remains that aflatoxicol is converted in the liver of an animal again into more toxic metabolites, such AFM_1, AFM_2. Hence, biotransformation strategies for feed and food decontamination, focus on a complete opening of the bisfuran ring system, as this would result in a complete loss of activity. Many microorganisms have been identified to be able to facilitate this complete degradation. However, the processes are time consuming (longer fermentation times required) and are currently only applied in traditionally fermented Asian foods, rather than as a practical procedure to decontaminate rapidly mycotoxin containing food and feed commodities [89,90].

Recent interest focus on fungal laccases, a class of copper-containing polyphenol oxidases, which are able to alter the α,β-unsaturated double bond between C-2 and C-6 of the furofuran ring in the AFB_1 molecule [91]. Such enzymes may become commercially available in the near future [92,93].

Opening of the lactone ring of ZEA

The core structure of ZEA is a resorcyclic acid lactone, which is rapidly modified by fungi and bacteria, such as *Cunninghamella bainieri* (ZEA to 2,4-dimethoxyl zearalenone),

Rhizopus arrhizus (ZEA to zearalenone 4-sulfate), *Thamidium elegans* and *Mucor bainieri* (ZEA to zearalenone-4-β-D-glucoside) [86,94–96]. These modified forms still contain the C-4 hydroxyl group of ZEA, and particular the sulphate and glucoside conjugates may be cleaved by other bacteria in the intestines, rendering the biologically active ZEA again. Previously it was reported that only the mycoparasite *Gliocladium roseum* (now called *Clonostachys rosea*) was able to open the lactone ring completely [86,97,98]. More recently, it has been observed that a lactonohydrolase encoded by the gene zhd101 in the fungus *Clonostachys rosea* is also able to cleave the lactone ring [99,100]. This finding suggests the possibility to use this enzyme in industrial processes.
Different from ZEA, patulin contains a polyketide lactone, which is hydrolysed by bacteria yielding deoxypatulinic acid, which is considered to be less toxic [101]. It has also been demonstrated that *S. cerevisiae* and other organisms have the potency to degrade patulin via opening the pyran ring resulting in the more polar metabolite described as ascladiol [102,103].

Hydrolysis of the amid bond of OTA
Microbiological hydrolysis of OTA cleaves the amide bond between the isocoumarin moiety and the L-β-phenylalanine side chain [104,105]. The resulting OTα is significantly less toxic. This hydrolysis step was first observed in rumen protozoa [106,107], but also by microorganisms from the intestinal tract of monogastric animal species. Comparable results have been obtained with different yeasts, including *Rhodotorula*, *Cryptococcus* as well as *Trichosporon mycotoxinivorans* and *Aureobasidium pullulans*, which can scavenge and degrade OTA [108–111]. Special attention and application has been given to *T. mycotoxinivorans* as this strain can biodegrade OTA as well as ZEA, expressing a broad set of exo-enzymes catalysing OTA-hydrolysis, as well as hydrolytic cleavage of the lactone ring of ZEA as described above [108,112].

Ester hydrolysis of fumonisin side chains
Fumonisins are not affected by the rumen flora, and only some fungi, such as *Exophiala* spp. are able to detoxify fumonisins [113] by expressing carboxylesterases that cleave the tricarballylate groups at C-14 and C-15. Thereafter, bacterial aminotransferases can further modify the side chain resulting in a 2-keto hydrolyzed FB_1 (HFB_1) as end product [114,115]. Such an enzymatic product is already licensed within the EU for the use in animal diets. HFB_1 is also formed non-enzymatically under strict alkaline conditions, and is recognized as a procedure that can be applied in food processing.

Microbial biotransformation of trichothecenes
A broad range of microbial biotransformation steps is described for individual trichothecenes considering self-protection mechanisms of fungi and plants by the conjugation of T-2 toxin or DON to glucosides of sulphates. These conjugation reactions result in less toxic metabolites, but the mammalian microbiota is able to hydrolyse

the conjugates resulting in the parent (toxic) mycotoxin. T-2 toxin, the most reactive trichothecene epoxide, may be converted by *Pseudomonas* spp. into HT-2 toxin and T-2 triol, which are slightly less toxic [116]. McCormick *et al.* [117] reported that different yeast species assigned to the *Trichomonascus* clade were able to detoxify T-2 toxin via three mechanisms of action, including acetylation in C-3 position, removal of isovaleryl group at C-8 position and glycosylation at the C-2 position. While T-2 toxin is subject to diverse biotransformation reactions, DON is more readily converted under anaerobic conditions by de-epoxidation into DOM-1, which is less toxic [118,119]. DOM-1 is a common metabolite of the intestinal microbiota. However, under aerobic conditions (and by Gram-positive bacteria), DON is apparently only transformed to its 3-epi-DON, which still contains the 12,13 epoxide group, and hence it is assumed that the 3-epi-DON is as toxic as DON [120]. This latter reaction would explain also the stability of DON in surface water (effluents from wheat and corn fields).

Conclusion

Mycotoxins are currently considered as the most prevalent contaminants of human food and animal feed, as they invade plants and plant-derived products at the pre- and post-harvest stage. Changes in agricultural techniques, such as minimal tillage and the increasing use of mono-cultures, as well as the global climate change have been discussed as possible reasons for the increase of mycotoxin contamination. The health concerns related to frequent exposure to one or more mycotoxins in the diet, comprise an increased risk for cancer, organ-specific lesions and the acceleration of inflammatory and immune-mediated disease conditions. In consideration of these important adverse health effects, there is a strong need to understand these pathophysiological alterations initiated by individual mycotoxins and mixtures thereof and to explore interventions strategies to reduce the oral bioavailability of mycotoxins or even inactivate mycotoxins with the help of food and feed additives. Various successful approaches have been described for the most important mycotoxins, but an overview of the available techniques and substances also indicates the clear limitations in term of efficacy and the risk for undesirable side effects. A typical example for very limited options in mycotoxin mitigation is the mycotoxin DON, which lacks any binding sites for chemical adsorbents, and can be enzymatically inactivated by de-epoxidation by a limited number of bacteria. As in Europe and many other parts of the world, the contamination of important staple foods, such as cereals and grains with DON was increasing rapidly during the last years, new concepts are needed to mitigate its adverse effects on human and animal health. New concepts for DON as well as other mycotoxins focus on the prevention of absorption as well as the protection of the intestinal barrier function, a common site of first action of most mycotoxins. The potential beneficial effects of organic polymers derived from yeast cells (mannans) or Gram-positive bacteria (peptidoglycans from Lactobacilli) and other polysaccharides from different sources (fructo-oligosaccharides (FOS) as well as

galacto-oligosaccharides (GOS)) are gaining increasing interest. Such compounds are able to sequester mycotoxins and exert at the same time a positive effect on gut health and the gut-associated immune system.

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Chapter 3

Deoxynivalenol and its modified forms: are there major differences?

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This chapter is submitted for publication

Abstract

Recent surveys indicate that human and animal diets are frequently exposed to one or more mycotoxins. The additional monitoring of human urine samples, confirmed an exceptional high exposure frequency for the *Fusarium* toxin deoxynivalenol (DON), albeit often at low concentrations. The first and major target in cases of dietary exposure is the intestinal barrier. Most of the studies have been conducted only with the parent compound DON, while human and animal exposure encompasses the acetylated fungal metabolites 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON) as well as the plant-derived DON-glucoside (DON3G) and the bacterial product DOM-1. In the current study we present investigations with the well-established Caco-2 cell model, comparing the effects of all these naturally occurring forms of DON on cell viability and markers of barrier integrity, such as trans epithelial electric resistance (TEER), the permeability of the marker Lucifer yellow, indicating paracellular transport, as well as the epithelial cell-derived pro-inflammatory chemokine CXCL8. Results show that the fungal metabolite 3ADON is slightly less potent to induce adverse effects on barrier integrity when compared to DON, whereas 15ADON appears to be even more potent than the parent DON. In contrast, the conjugate DON3G and the bacterial de-epoxide DOM-1 exerted no measurable adverse effects on the intestinal barrier *in vitro*. In a parallel series of experiments, it could also be demonstrated that galacto-oligosaccharides (GOS) are able to protect epithelial cells against DON and its acetylated forms. Particularly these protective effects of GOS are of clinical relevance, as it suggests beneficial effects of GOS as food additives not only in infant diets, but also in the protection of vulnerable segments of the human population against adverse effects of DON, which may contaminate important grain-based common food supplies.

Introduction

Trichothecenes comprise a large family of structurally related mycotoxins produced by various *Fusarium* species. Chemically, trichothecenes belong to the group of sesquiterpenoids and share a 9, 10 double bond and the 12, 13 epoxide group, the latter being considered to be critical for their toxicity (Figure 1) [1–3]. The mycotoxin deoxynivalenol (DON), mainly produced by *Fusarium graminearum* and *Fusarium culmorum*, is one of the main representative of the type B trichothecenes and is currently among the most prevalent and important contaminants of cereals and cereal-based products [4–6]. Recently initiated monitoring of urine samples for the presence of DON confirms the high rate of exposure, albeit in many cases at low levels [5,7,8].
The first target of DON toxicity seems to be the intestinal tract, as DON is able to impair the intestinal barrier by affecting the expression and assembly of tight junctions and initiating an inflammatory response [9]. Subsequently, DON can increase the paracellular transport of luminal antigens and even pathogens *in vitro* and *in vivo* [10–12], and in humans DON may even stimulate the development of allergies, including whey allergy as demonstrated in a murine model [13].

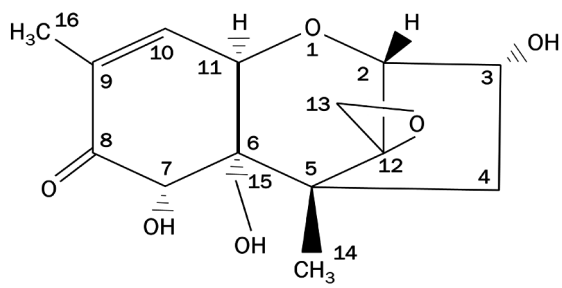

Figure 1. Molecular structure of deoxynivalenol

Fusaria produce not only DON, but also two prominent acetylated derivatives of DON, the 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON) [5,14–16]. Nowadays *F. graminearum* genotypes producing all 3 forms (DON, 3ADON and 15ADON) have been identified [17]. Plants in turn aim to protect their tissues conjugating free DON to one or more molecules of glucose of which DON-3-β-d-glucoside (DON3G) is the most frequently measured conjugate [14,18,19]. The glycosylation changes the physico-chemical properties of DON significantly, and hence such plant glucosides remained long been undetected and have been described as “masked” mycotoxins, as they escape common extraction and detection methods validated for the determination of DON [20,21]. DON3G is detectable in approximately 4-12% of the respective DON concentration in maize and wheat samples [18]. Upon ingestion with food, the glucoside binding can be cleaved readily by bacterial glucosidases. Gratz *et al.* [22] reported that mixed human fecal microbiota are capable of hydrolyzing 80% of DON3G in 4 h.

Comparable data for other DON glucosides are not available, but it is generally assumed that liberated DON contributes to overall human and animal exposure [14,18,19,23].
The intestinal microbiome does not only liberate DON from plant material, but is also able to convert DON to its de-epoxide, denoted DOM-1 [3,24]. Although earlier studies failed to detect significant amounts of DOM-1 in human blood samples, DOM-glucuronides have been detected in human urine samples and recently in urine samples of children as well. These findings indicate that part of the formed DOM-1 is absorbed and subsequently excreted via the kidneys [7]. All the above mentioned derivatives and metabolites of DON are summarized in Table 1.
The aim of the current study was a direct comparison of the different DON-derivatives in a standardized Caco-2 cell model that is widely accepted to study direct effects of drugs and toxins on the epithelial barrier. In parallel, we tested the protective effect of a defined formulation of non-digestible oligosaccharides under the same experiment conditions. These galacto-oligosaccharides (GOS) are produced from lactose and are widely used in infant diets due to their prebiotic and immuno-modulating properties [25–30]. In previous *in vitro* and *in vivo* experiments we already demonstrated that GOS are able to protect from the breakdown of the intestinal barrier following exposure to DON [28]. This protective effect of GOS concerned not only the maintenance of barrier integrity measured by trans-epithelial electrical resistance (TEER) and paracellular transport of marker molecules, such as Lucifer yellow (LY), but also an anti-inflammatory effect, preventing the DON-induced increase in the secretion of the pro-inflammatory chemokine CXCL8.

Table 1. DON and its common modified forms

Common DON derivatives and metabolites	Origin	Structure (Figure 1)
DON	Fungi	(3α,7α)-3,7,15-trihydroxy-12,13-epoxy-trichothec-9-en-8-one
3-acetyl-DON (3ADON)	Fungi	- C_2H_3O (Acetyl)
15-acetyl-DON (15ADON)	Fungi	- C_2H_3O (Acetyl)
De-epoxy-DON (DOM-1)	Bacterial metabolite of DON	- CH_2 (at C12)
DON-3-O-glucoside (DON3G)	Plant conjugates of DON	- $C_6H_{11}O_5$ (Glucoside)

Materials and Methods

Caco-2 culture

The human intestinal Caco-2 cell line was obtained from American Type Tissue Collection (passages 5–19; HTB-37; Manassas, VA, USA). The cells were cultured in 75-cm^2 culture flasks (Greiner Bio-One, Frickenhausen, Germany) and the Dulbecco's modified Eagle's minimum essential medium (DMEM), supplemented with 25 mM HEPES, 4.5 g/l glucose (Gibco, Invitrogen, Carlsbad, CA, USA), 10% (v/v) inactivated fetal calf serum (FCS) (Gibco), glutamine (2 mM, Biocambrex, Verviers, Belgium), 1% (v/v) nonessential amino acids (Gibco), penicillin (100 U/ml)/streptomycin (100 µg/ml) was utilized as medium for the growing cells. The cells were preserved in an incubator to provide them with the optimum moisture and temperature (humidified atmosphere of 95% air and 5% CO_2 at 37˚C). The cells were cultured in a transwell system according to the protocol described by Akbari *et al.* [10].

Mycotoxins

DON, 15ADON, 3ADON and DOM-1 (Sigma-Aldrich, St Louis, MO, USA) and DON3G (Romer Labs GmbH, Tulln, Austria) were diluted in absolute ethanol (99.9%; JT Baker) to prepare the stock solution. Working dilutions were prepared in cell culture medium.

GOS

The commercially available Vivinal® GOS syrup (FrieslandCampina Domo, Borculo, The Netherlands), comprising 45% GOS with a DP (degree of polymerization) of 2-8, 16% free lactose, 14% glucose, and 25% water, was used. Dilutions of GOS (1% and 2%) were prepared in complete cell culture medium. Previous experiments confirmed that GOS did not induce any cytotoxicity in Caco-2 cells at the selected concentrations [28].

Co-exposure experiments with mycotoxins and GOS

The Caco-2 cells were grown on transwell culture inserts and exposed from the apical and basolateral side to different concentrations of DON, 15ADON, 3ADON, DON3G and DOM-1 for 24 h in a concentration range of 2.1 to 8.4 µM.

Additionally, in parallel experiments, Caco-2 cells were pretreated with GOS (1% and 2%) for 24 h (added to both the apical and basolateral compartments) and then challenged by either 4.2 µM DON or 4.2 µM DON fungal derivatives (3ADON and 15ADON) from apical and basolateral side in the presence of GOS for the next 24 h according to the protocol described in our previous studies [10,28].

Lactate dehydrogenase (LDH) assay

Caco-2 cells were exposed to DON, 3ADON, 15ADON, DON3G and DOM-1 (2.1, 4.2, 8.4 µM) for 24 h and the cytotoxicity of the mycotoxins was evaluated by measuring the LDH release in the supernatant of apical and basolateral compartments using the CytoTox 96 non-radioactive cytotoxicity assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

TEER measurement

The Caco-2 monolayer integrity was assessed by TEER measurement utilizing Millicell-ERS volt-ohm meter (Millipore) at different time points (4, 8, 12 and 24 h) by using increasing concentrations of DON, 3ADON, 15ADON, DON3G and DOM-1 (2.1 to 8.4 µM). In line with these experiments the cells were pre-incubated with GOS (1% and 2%) for 24 h followed by DON, 3ADON and 15ADON challenge (4.2 µM) for 24 h and TEER values were recorded at different time points (8 h, 12 h and 24 h).

Paracellular permeability assay

For the determination of the paracellular permeability, the flux of Lucifer yellow (LY, molecular mass of 0.457 kDa, Sigma Chemical Co, St Luis, Mo, USA) was evaluated. The transport studies were conducted by adding 16 µg/ml of LY to the apical compartment (350 µl) of the transwell inserts for 4 h and measuring the paracellular flux to the basolateral compartment by quantifying the fluorescence intensity using a fluorometer (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 410 and 520 nm respectively.

CXCL8 (chemokine CXC motif ligand)

The amount of CXCL8 release by Caco-2 cells into the apical and basolateral compartments of the transwell inserts was quantitatively determined by the human IL-8 ELISA Set (BD Biosciences, San Diego, CA, USA) in accordance with the manufacturer's instructions.

Statistical analysis

The results from all mycotoxin comparison studies were expressed as mean ± SEM of 3 independent wells (3 wells/condition), whereas the results of all mycotoxin studies in combination with GOS were expressed as mean ± SEM of 4 independent experiments (n=4), each performed in triplicate (3 wells/condition). Differences between results were statistically evaluated using One-way ANOVA with Bonferroni post-hoc test.

Results

Comparison of LDH leakage induced by DON and its derivatives and metabolites

Non-specific cytotoxicity was determined by measuring the LDH leakage from differentiated Caco-2 cells. Results are presented individually for the apical and basolateral compartment. LDH leakage measured at the apical compartment following exposure to 8.4 µM DON or 15ADON was comparable, whereas no effect of 3ADON on the LDH release into the apical compartment was observed (Figure 2A and 2C) in comparison to control. In contrast, 3ADON (8.4 µM) and 15ADON (4.2 and 8.4 µM) significantly increased the LDH release into the basolateral compartment (Figure 2B and 2D), whereas DON did not significantly affect the LDH release into the basolateral compartment.

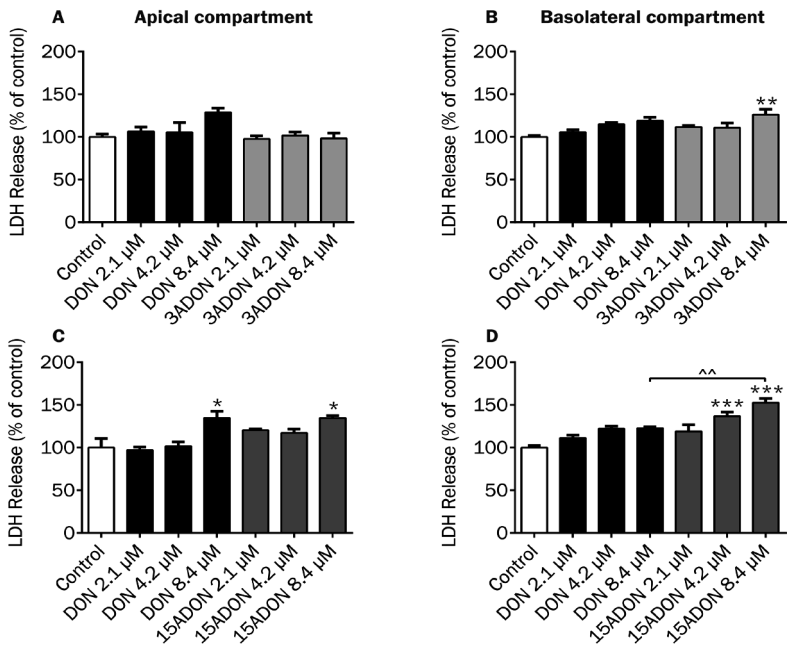

Figure 2. Cytotoxic effects of DON, and its acetylated derivatives in Caco-2 cells. Differentiated Caco-2 cells on inserts were exposed from the apical and basolateral compartment to increasing DON, 3ADON and 15ADON concentrations (2.1, 4.2, 8.4 µM) for 24 h followed by evaluation of LDH release into the apical (A and C) and basolateral (B and D) compartment. Results are expressed as a percentage of LDH released by the control group as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; significantly different from control group; ^^P < 0.01; significantly different from corresponding DON group.

Comparable experiments with DON3G and DOM-1 using the same concentration range, did not result in any significant changes in LDH leakage compared to control (Figure 3A, 3B, 3C and 3D). Even at a higher concentration (16.8 µM), DON3G and DOM-1 did not significantly increase the LDH leakage (data not shown).

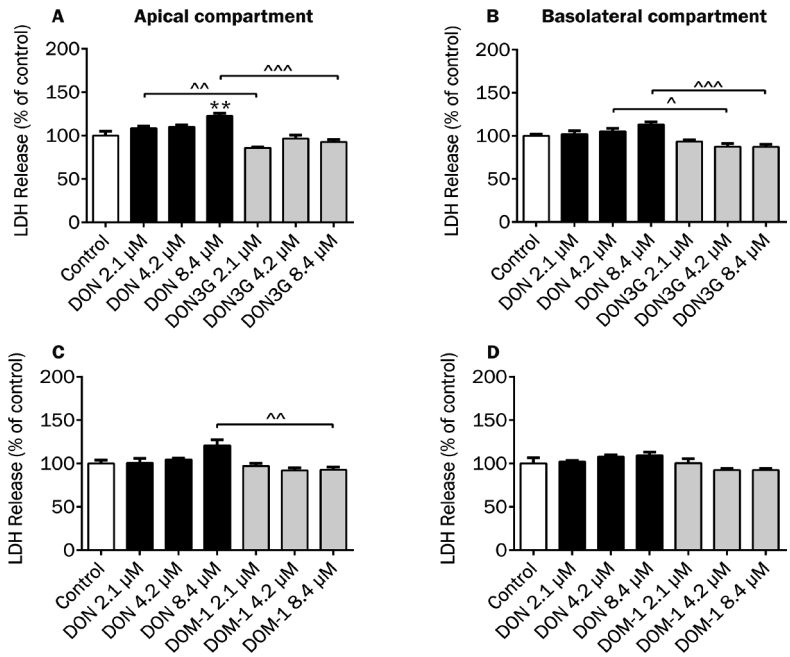

Figure 3. Cytotoxic effects of DON, DON3G and DOM-1 in Caco-2 cells. Differentiated Caco-2 cells on inserts were exposed from the apical and basolateral compartment to increasing DON, DON3G and DOM-1 concentrations (2.1, 4.2, 8.4 µM) for 24 h followed by evaluation of LDH release into the apical (A and C) and basolateral (B and D) compartment. Results are expressed as a percentage of LDH released by the control group as mean ± SEM. **P < 0.01; significantly different from control group; ^P < 0.05, ^^P < 0.01, ^^^P < 0.001 significantly different from corresponding DON group.

Comparison of changes in trans-epithelial resistance induced by DON and its derivatives and metabolites

TEER is a commonly used marker for the integrity of the tight cellular monolayer. In the current experiments, 3ADON and 15ADON concentration-dependently decreased the TEER values comparable to the effects of DON effects at different time points (Figure 4A and 4C). However, 3ADON is slightly less potent to induce a decrease in TEER values compared to DON, whereas 2.1 µM 15ADON appears to be even more potent in decreasing TEER values compared to 2.1 µM DON (12 h).

In the second series of experiments, a comparison between the effects of DON and DON3G or DOM-1 was made and both modified forms did not show any effect on TEER in the tested concentration range (Figure 5A and 5C).

Comparison of changes in paracellular transport across the monolayer induced by DON and its derivatives and metabolites

The paracellular transport of LY across the Caco-2 monolayer was measured as a functional parameter of an impaired barrier function. As expected on the basis of the TEER measurements, 3ADON and 15ADON concentration dependently increased LY permeability in a comparable manner as DON (Figure 4B and 4D), albeit minor differences were observed as 3ADON was less effective in increasing the Caco-2 monolayer permeability compared to DON, whereas 2.1 µM 15ADON had a more pronounced effect on LY translocation than DON (Figure 4B and 4D). Exposure of cells to DON3G and DOM-1 did not result in measurable changes of LY transport from the apical to basolateral compartment (Figures 5B and 5D).

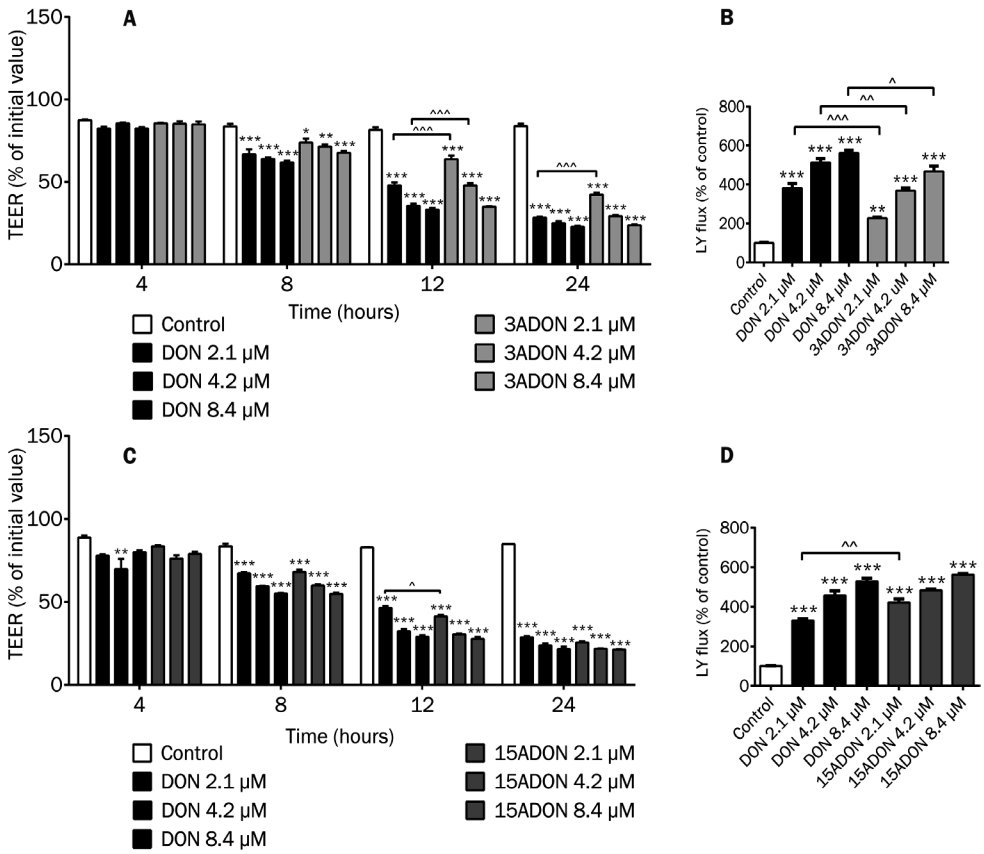

Figure 4. Effects of DON and its acetylated derivatives on TEER and LY translocation. Differentiated Caco-2 cells on inserts were exposed from the apical and basolateral compartment to increasing concentrations of DON, 3ADON and 15ADON for 24 h and TEER values were measured during different time points (A and C). Subsequently, LY translocation from the apical to the basolateral compartment was measured (B and D). Results are expressed as percentage of initial value (TEER values) or percentage of control group (LY translocation) as mean ± SEM. *P <0.05, **P < 0.01, ***P < 0.001; significantly different from corresponding control group, ^P < 0.05, ^^P < 0.01, ^^^P < 0.001; significantly different from corresponding DON group.

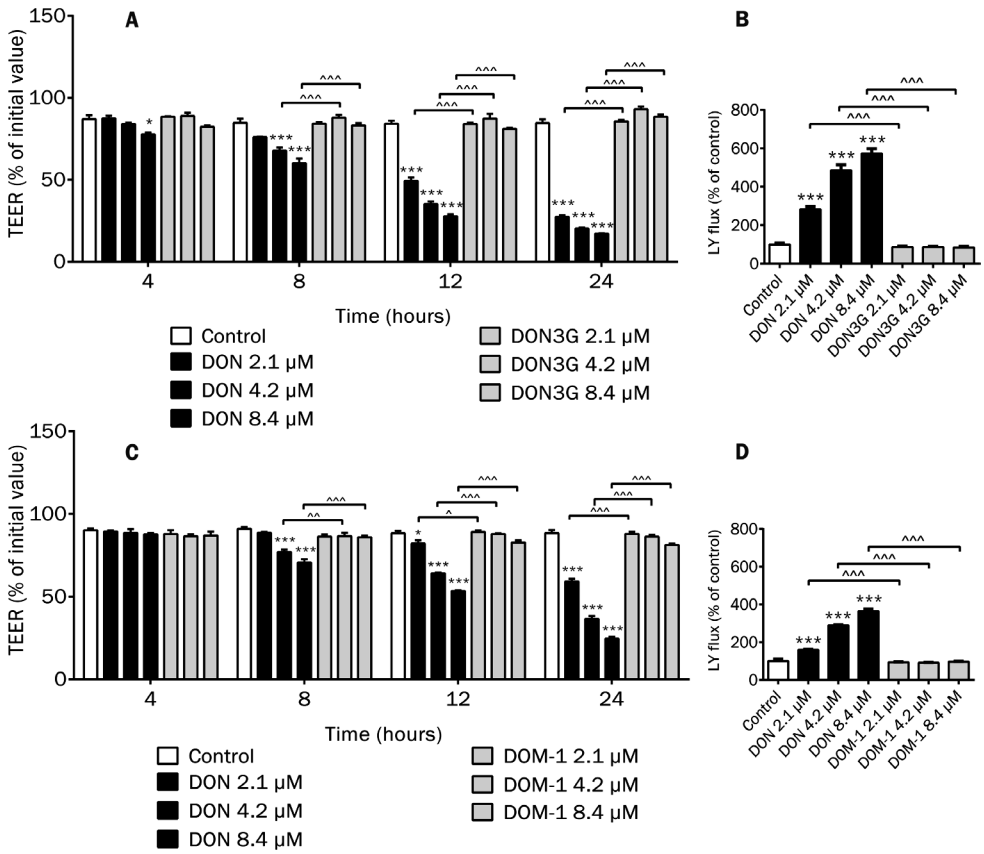

Figure 5. Effects of DON, DON3G and DOM-1 on TEER and LY translocation. Differentiated Caco-2 cells on inserts were exposed from the apical and basolateral compartment to DON, DON3G and DOM-1 for 24 h and TEER values were measured during different time points (A and C). Subsequently, LY translocation from the apical compartment to the basolateral compartment was measured (B and D). Results are expressed as percentage of initial value (TEER values) or percentage of control group (LY translocation) as mean ± SEM. *P < 0.05, ***P < 0.001; significantly different from corresponding control group, ^P < 0.05, ^^P < 0.01, ^^^P < 0.001 significantly different from corresponding DON group.

Comparison of changes in CXCL8 secretion induced by DON and its derivatives and metabolites

DON as well as 3- and 15ADON concentration-dependently induced the secretion of the pro-inflammatory chemokine CXCL8 in both apical and basolateral compartments of the Caco-2 cell model (Figure 6A, 6B, 6C and 6D). For all three DON forms, the CXCL8 excretion was higher at the apical site. No further remarkable differences were observed between the individual DON forms except that 2.1 µM 3ADON was less potent in stimulating the CXCL8 release into the basolateral compartment compared to 2.1 µM DON (Figure 6B).

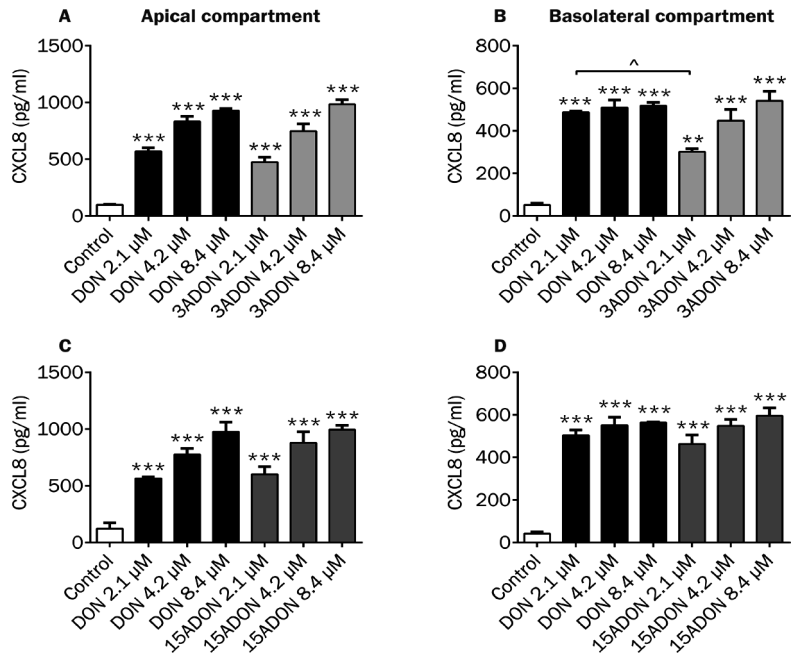

Figure 6. Effects of DON, and its acetylated derivatives on CXCL8 secretion. Differentiated Caco-2 cells on inserts were exposed from the apical and basolateral compartment to DON, 3ADON, 15ADON for 24 h followed by evaluation of CXCL8 secretion into the apical (A and C) and basolateral (B and D) compartment. Results are expressed as pg/ml as mean ± SEM. **P < 0.01, ***P < 0.001; significantly different from control group; ^P < 0.05 significantly different from corresponding DON group.

Comparable experiments were conducted with DON3G and DOM-1, again without any significant response on the CXCL8 secretion, confirming that these forms of DON are much less active than the parent DON and its acetylated forms (Figure 7A, 7B, 7C and 7D).

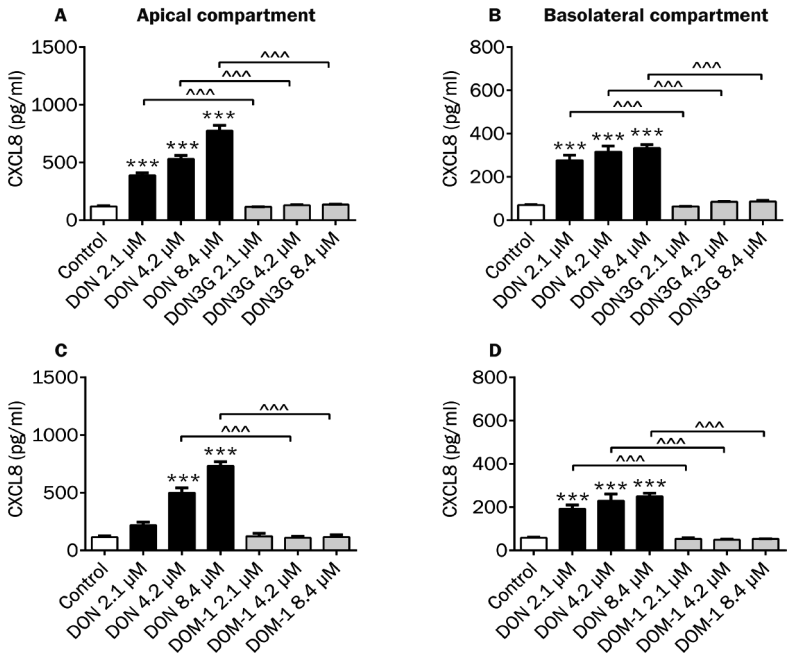

Figure 7. Effects of DON, DON3G and DOM-1 on CXCL8 secretion. Differentiated Caco-2 cells on inserts were exposed from the apical and basolateral compartment to DON, DON3G and DOM-1 for 24 h followed by evaluation of CXCL8 secretion into the apical (A and C) and basolateral (B and D) compartment. Results are expressed as pg/ml as mean ± SEM. ***P < 0.001; significantly different from control group; ^^^P < 0.001 significantly different from corresponding DON group.

GOS prevent and suppress the barrier disrupting and pro-inflammatory effects exerted by DON and its acetylated derivatives

In consideration of our previous results, we finally wanted to confirm that GOS are also able to suppress effects of DON and its biologically active acetylated forms. In Figure 8 and 9, composite panels summarizing the results obtained with TEER measurement, LY permeability and CXCL8 secretion are presented. Compared to the effects of GOS on the DON-induced TEER decrease and LY increase (Figure 8A and 8B), GOS 2% was also able to attenuate the 3ADON and 15ADON-induced TEER increase at 12 h and 24 h (Figure 8C and 8E). The GOS preventive effects in 3ADON- and 15ADON-stimulated cells after 24 h stimulation were more pronounced compared to 12 h stimulation (Figure 8C and 8E). The main finding of these experiments is that 2% GOS was equally effective in the suppression of 3ADON and 15ADON effects as compared to DON. Comparable results were observed for the CXCL8 release (Figure 9).

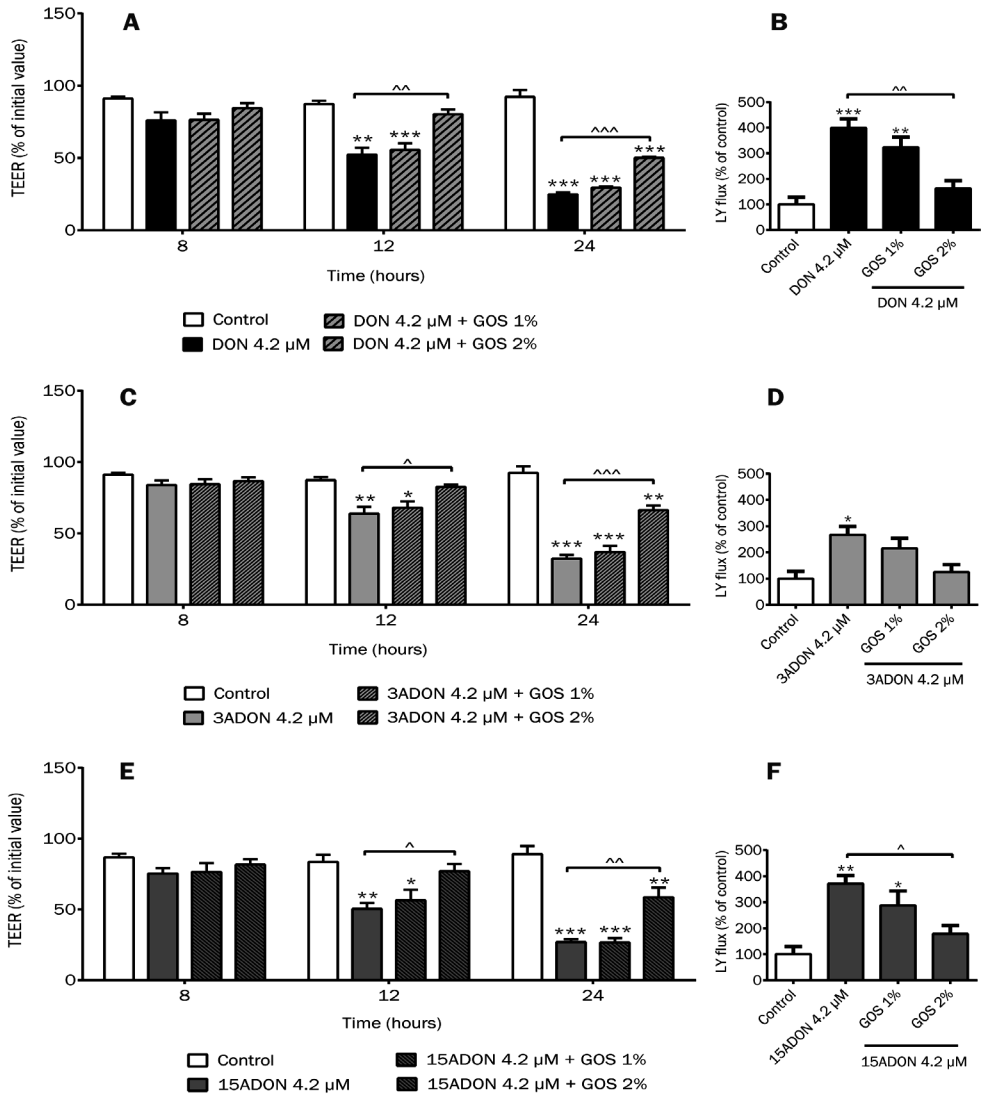

Figure 8. Effects of GOS on the disruption of intestinal barrier induced by DON and its acetylated derivatives. Differentiated Caco-2 cells on inserts were pretreated from the apical and basolateral compartment with GOS for 24 h followed by DON (A and B), 3ADON (C and D) and 15ADON (E and F) exposure for 24 h in a concentration of 4.2 µM and TEER values were measured during different time points. Subsequently, the LY translocation from the apical to the basolateral compartment was measured. Results are expressed as percentage of initial value (TEER values) or percentage of control group (LY translocation) as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; significantly different from corresponding control group. ^P < 0.05, ^^P < 0.01, ^^^P < 0.001; significantly different from corresponding DON, 3ADON and 15ADON group.

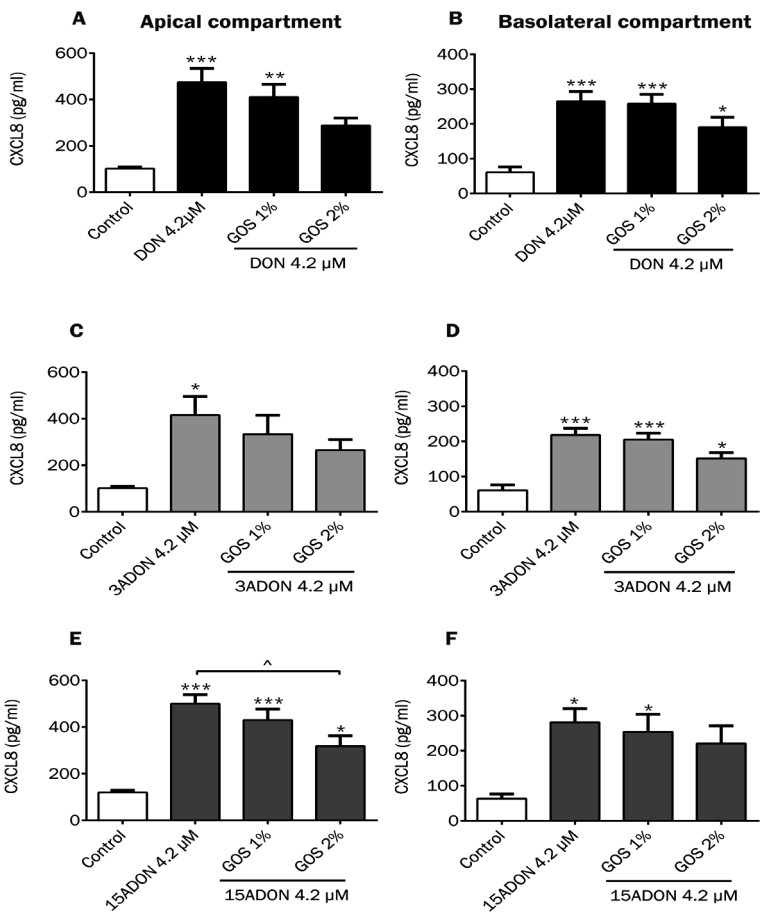

Figure 9. Effects of GOS on the CXCL8 release induced by DON and its acetylatedderivatives. Differentiated Caco-2 cells on inserts were pretreated from the apical and basolateral compartment with GOS for 24 h followed by DON (A and B), 3ADON (C and D) and 15ADON (E and F) exposure for 24 h and secretion of CXCL8 into the apical and basolateral compartment was measured. Results are expressed as pg/ml as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; significantly different from control group; ^P < 0.05 significantly different from 15ADON group.

Discussion

Considering the diverse toxic effects of the trichothecene mycotoxin DON and its common occurrence in small grains, particular wheat, as well as its stability during food and feed processing, DON constitutes an increasing health concern for humans and animals [15,31,32]. In addition to the parent DON molecule, humans and animals may be exposed to additional fungal derivatives of DON, such as 3ADON and 15ADON. Next to these fungal derivatives, human and animals are also exposed to plant derived DON-glucosides, including DON3G. These conjugates are expected to have a low toxicity, but may contribute to overall exposure [5,19,20,23].

The first aim of the current study was to compare the different modified forms of DON in a well-established Caco-2 cell model. The selected endpoints of toxicity were barrier function measured by TEER and paracellular transport of the marker Lucifer yellow, as well as the DON-induced inflammatory response, measured as CXCL8 release.

The cytotoxicity of DON derivatives and metabolites was measured by means of LDH leakage following exposure of Caco-2 cell monolayers from the basolateral as well as the apical side. These cytotoxicity results demonstrated that DON and its acetylated derivatives 3ADON and 15ADON induced no immediate cytotoxicity up to a concentration of 4.2 µM. Akbari *et al.* [10] also reported that Caco-2 cells are resilient to DON in a concentration range up to 12.5 µM following exposure for 24 h. Previous studies demonstrated no significant effects on Caco-2 cell viability in a concentration range of 0.337 to 33.7 µM DON (48 h) and 0.337 to 33.7 µM DON acetylated derivatives (6 h) [16,33]. For 3ADON a significant increase in extracellular LDH was measured at the highest concentration (8.4 µM) tested, whereas for 15ADON significantly increased values, exceeding even those measured for DON, were observed at 4.2 and 8.4 µM. The latter finding might indicate a more rapid cellular uptake of the acetylated derivatives.

In contrast, neither DON3G nor DOM-1 exerted any LDH leakage over the entire concentration range up to the highest concentration of 8.4 µM. In addition, even a higher concentration (16.8 µM) of DON3G and DOM-1 did not impair cell viability as indicated by the LDH release (data not shown). The results are in line with recently published data observing also no cytotoxic effects of DON3G (0.05 µM to 10 µM) after 48 h exposure in proliferating Caco-2 cells, whereas differentiated Caco-2 cells showed no cytotoxicity up to 100 µM for 8 days [34].

Decrease in TEER values and increase in LY permeability as assessed in the current study provide strong evidences that DON and its acetylated derivatives (3ADON and 15ADON) have the potency to alter the permeability of Caco-2 cells. Reduction in TEER values following exposure to DON have been reported in several studies with different cell lines, including Caco-2 [10–12,35], IPEC-1 cells [12] and IPEC-J2 [36], but studies with DON derivatives are scarce. It has been reported that 15ADON is more potent to affect TEER values in porcine intestinal columnar epithelial cells (IPEC-1) [37] and in Caco-2 cells [16] compared to DON and 3ADON. Contradictory to these studies the

current results demonstrated that DON and 15ADON induced similar effects on TEER, except the 2.1 µM 15ADON which appears to be more potent than DON after 12 h exposure. The adverse effects on the epithelial integrity of 3ADON were less pronounced compared to DON.

These differences between the individual studies might be due to the different cell lines (IPEC-1 *vs* Caco-2 cells), exposure route (apical exposure *vs* exposure from both sides in our study) as well as the duration of exposure [16,37].

As hypothesized, DON3G had no effect on TEER values, which is in line with previous results in which Caco-2 cells were exposed to 10 µM DON3G for 8 days [34]. In parallel, DOM-1 did not influence TEER values over the entire concentration range tested.

The TEER results of the current study are compatible with the LY permeability data. Increasing concentrations of DON, 15ADON and 3ADON resulted in enhanced paracellular translocation of the marker LY from the apical to the basolateral site, and a direct comparison revealed that the effect of 3ADON was less pronounced than that of 15ADON, the latter closely resembling the effects of DON.

Again, as expected DON3G and DOM-1, which did not affect TEER vales, did also not result in an increase in the paracellular transport of LY.

Due to the fact that CXCL8 is pivotal for the progress of most local intestinal inflammatory reactions [38], the CXCL8 release into the supernatant of Caco-2 cells exposed to DON and its derivatives and metabolites was examined. The results resemble to a large extent the results of the markers of barrier integrity. A highly significant increase in CXCL8 was measured for DON and both acetylated forms. The most pronounced increase in CXCL8 was measured at the apical site. Our observations of the DON-induced CXCL8 release are in agreement with previous studies, where DON had the potency to induce CXCL8 secretion by Caco-2 cells [11,39].

The results of the comparable experiment with DON3G and DOM-1 confirmed that DON3G had no effect on CXCL8 secretion, which was previously reported by measuring the CXCL8 mRNA expression [34].

Taken together, our current results confirm that the DON3G has no significant biological activity on the tested functional parameters of the intestinal barrier. Recent elegant *in silico* and *in vitro* experiments indicated that DON3G is apparently poorly absorbed by intestinal cells and fails to bind to the A-site of the ribosome peptidyl transferase center, and hence is not able to induce the activation of mitogen activated protein kinases (MAPK), which is the main pathway resulting in the typical ribotoxic stress considered as main molecular mechanism of DON-induced cell injury and inflammation [34].

From numerous other plant secondary metabolites that are present as glucosides, it is known that the oral bioavailability is low, which also applies to DON3G [40]. However when these glucosides reach the lower parts of the intestine with a higher microbiota density, the glucosides are cleaved to a large extend by bacterial glucosidases, which leads to the release of the parent DON molecule.

The liberated DON contributes to human and animal exposure particularly in the large

intestine, from which it also can be absorbed [34]. The actual concentration of free DON that reaches via this secondary route to the systemic circulation is expected to be highly variable, as bacterial de-epoxidases can rapidly convert the released DON to DOM-1, which is significantly less toxic [41]. DOM-1 has also been detected in human urine samples [42,43].

Comparing DON and its acetylated forms, it has been suggested that acetylated DON-forms are more rapidly absorbed from the intestines [16], a process that would be initiated by a more rapid uptake by intestinal cells. At the cellular level, DON inhibits protein synthesis via triggering a ribotoxic stress response through its high binding affinity to the peptidyl transferase region of the ribosome leading to the activation of the MAPK as mentioned above [9,44]. Pinton *et al.* [37] showed differences in toxicity between DON, 3ADON and 15ADON related to their ability to activate the MAPK and concluded that 15ADON is a more potent inducer of MAPKs. Comparable effects were also confirmed *in vivo*, where 15ADON induced more pronounced changes in the intestinal architecture [37].

In recent approaches towards the establishment of health-based guidance levels, an integral approach for the toxicological assessment of the parent mycotoxin and its modified forms (the general term for all identified metabolites) is applied. Such an approach was recently published by the European Food Safety Authority (EFSA) regarding the mycotoxin zearalenone and its modified forms, resulting in a so-called group health based guidance level [45]. Applying this approach to DON, the current results suggest that a toxic equivalent factor of > 1 should be used for 15ADON co-occurring with DON in food materials, whereas the equivalent factor for 3ADON would be slightly < 1. For DON3G and DOM-1 toxic equivalent factors would not be necessary (or close to zero), but the microbiota-dependent cleavage of DON3G needs to be considered in the overall exposure assessment.

The second objective of this study was to investigate whether or not the already demonstrated protective effect of GOS on the DON-induced impairment of intestinal barrier function, can also be expected to apply for other biologically active forms of DON, such as 3ADON and 15ADON. The protective effects of GOS on DON-induced breakdown of the barrier integrity had been previously demonstrated by our group in the same Caco-2 cell model [28]. The obtained results confirm this protective effect of GOS (2%) not only to DON but also against 3ADON and 15ADON.

Recent epidemiological data suggest an extremely high frequency of exposure, but concentrations in human food still remain low and within the statutory limits. However, even at such low food concentrations, the effects of DON on intestinal barrier integrity remains of concern, as this might result in chronic inflammatory disease, including inflammatory bowel disease in humans, and in an increased allergic response to certain food allergens. This is of particular importance, as our group had already demonstrated that DON increased the risk of whey allergy [13].

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Chapter 4

Deoxynivalenol impairs weight gain and affects markers of gut health after low-dose, short-term exposure of growing pigs

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This chapter is published in Toxins 2015; 7(6): 2071-2095

Abstract

Deoxynivalenol (DON) is one of the major mycotoxins produced by *Fusarium* fungi, and exposure to this mycotoxin requires an assessment of the potential adverse effects, even at low toxin levels. The aim of this study was to investigate the effects of a short-term, low dose DON exposure on various gut health parameters in pigs. Piglets received a commercial feed or the same feed contaminated with DON (0.9 mg/kg feed) for 10 days, and two hours after a DON bolus (0.28 mg/kg BW), weight gain was determined and samples of different segments of the intestine were collected. Even the selected low dose of DON in the diet negatively affected weight gain and induced histomorphological alterations in the duodenum and jejunum. The mRNA expression of different tight junction proteins, especially occludin, of inflammatory markers, like Interleukin-1 beta and Interleukin-10 and the oxidative stress marker heme-oxygenase1, were affected along the intestine by low levels of DON in the diet. Taken together, our results indicate that even after low-level exposure to DON, which has been generally considered as acceptable in animal feeds, clinically-relevant changes are measurable in markers of gut health and integrity.

Introduction

Deoxynivalenol (DON, vomitoxin) is the most frequently-occurring type B trichothecene produced by several field fungi, including *Fusarium graminearum* and *Fusarium culmorum*, and is commonly found in cereals and grains, particularly wheat, barley and maize, in areas with a moderate climate. The effects of DON exposure on different animal species related to the concentration and duration of exposure has been described in several reviews [1–4]. As DON is heat-stable, it resists common processing procedures during feed manufacturing [5]. Exposure to DON can induce gastrointestinal inflammation and necrosis within the intestinal tract and disturbs the gut barrier function [3,6,7]. In addition, DON is able to cause alterations in brain functions [8,9], induces vomiting (hence the name) and negatively affects the growth hormone production [10]. *In vivo* animal studies and practical data support the hypothesis that moderate levels of exposure to DON result in temporary feed refusal, lower feed intake accompanied with a reduction in weight gain, whereas at higher doses of DON, clinical signs of intoxication include emesis, leukocytosis, hemorrhage and even circulatory shock [4,9,11]. *In vivo* and *in vitro* studies present evidence that DON exerts an array of effects at the cellular level, including an increase in pro-inflammatory gene expression [12,13], impairment of cell division, proliferation, differentiation and cell membrane integrity, as well as induction of apoptosis [7,14]. There are obvious species variations in the susceptibility to DON, and pigs show the highest sensitivity to DON. This is in agreement with the high oral bioavailability of DON in pigs, which precedes the detoxification of DON by the flora of the large intestines to de-epoxy DON (DOM), which is less toxic [4,15]. The major concerns related to low-dose exposure to DON are its direct effects on the intestinal barrier and the intestinal immune system, as they may have a significant impact on pig health and performance [3,16,17], as also indicated in the summary of recent *in vivo* piglet studies, as presented in Table 1. The recommended maximum acceptable level for DON according to European Commission Recommendation 2006/576/EC is 0.9 mg/kg feed. Considering the available literature and the EU recommendation, the current study focused on DON-associated effects in the intestinal tract of growing pigs with the aim to identify sub-clinical alterations that might impair animal performance and, hence, serve as biomarkers of low-dose exposure to DON. To this end, pigs were given DON (0.9 mg/kg feed) in the diet for a period of 10 days, whereafter various gut health parameters were investigated.

Table 1. Summary of the *in vivo* pig studies related to the effects of dietary deoxynivalenol (DON) on the intestine.

DON	Trial period	Biomarker	DON effects	Ref.
4 mg/kg feed	37 days	Oxidative stress markers in blood (catalase (CAT), total antioxidant capacity (T-AOC), hydrogen peroxide (H_2O_2), nitric oxide (NO), maleic dialdehyde (MDA) and diamine oxidase (DAO)), kidney, liver and small intestine (H_2O_2, MDA and DAO) Intestinal morphology	DON induced oxidative stress DON increased intestinal permeability DON inhibited protein synthesis and cell proliferation	[18]
2.9 mg/kg feed	1 week	DON transport study	Dietary DON affected the jejunal transport of DON	[19]
3.1 mg/kg feed	37 days	Crypt depth Intestinal cell proliferation Immunofluorescence staining zona occludens protein-1 (ZO-1) and β-catenin	No effect on crypt depth No effect on epithelial cell proliferation No effect on apical junction proteins	[20]
2.3 mg/kg feed	35 days	Intestinal morphology/ histological score jejunum Mitogen activated protein kinases (MAPK) expression in jejunum	DON induced histological lesions DON activated MAPK extracellular-signal-regulated kinases 1/2 (ERK1/2) and p38	[21]
2.2 – 2.9 mg/ kg feed	11 weeks	Composition and perforation of the basement membrane of intestinal villi Presence of $CD16^+$ cells or their dendrites in the epithelium	DON increased the pore number in jejunum DON increased the number of $CD16^+$ cells in the epithelium of the jejunum	[22]
4 mg/kg feed	30 days	Intestinal morphology Intestinal function	DON enhanced intestinal permeability, damaged villi, caused epithelial cell apoptosis and inhibited protein synthesis	[23]
3 mg/kg feed	5 weeks	Intestinal morphology, Intestinal cytokine expression Tight and adherens junction protein expression (occludin (OCLN), E-cadherin)	DON induced atrophy and fusion of villi DON decreased villi height and cell proliferation in the jejunum. DON reduced number of goblet cells and lymphocytes DON induced up regulation of cytokine expression in jejunum and ileum. DON reduced the expression of E-cadherin and OCLN in ileum	[16]

3 mg/kg feed	10 weeks	Growth performance Histo-morphometric and immuno-fluorescence investigations of small intestinal epithelium	DON decreased the feed intake (grower) DON increased the crypt depth in jejunum No effect on villus height and ZO-1 expression in jejunum and ileum	[24]
2.29 mg/kg feed	4 weeks	Intestinal morphology/ histological scores	DON induced atrophy and villus fusion, necrotic debris and areas of enterocytes lyses DON caused 15% lower histological scores in jejunum	[25]
2.85 mg/kg feed	5 weeks	Claudin-4 (CLDN4) expression (Western Blot, immunofluorscence staining) in jejunum	DON reduced CLDN4 expression in jejunum	[26]
2.8 mg/kg feed	4 weeks	Growth performance, intestinal microflora	DON reduced the daily weight gain (first week) Moderate effect on cultivable bacteria in the intestine	[27]
1.2–2 mg/kg feed	84 days	Gene expression in ileum	DON induced a down-regulation interleukin-1 beta (IL-1β) and IL-8 expression in ileum	[28]
12 µg/kg BW/day	42 days	Absorption, accumulation and final presence of DON in the gastrointestinal tract	Presence of DON in intestinal tissues: DON concentrations in small intestine ranged from 7.2 (in the duodenum) to 18.6 ng/g (in the ileum) and in large intestine from 1.8 (in transverse the colon) to 23.0 ng/g (in the caecum)	[29]
1.5 mg/kg feed	28 days	Weight gain, histological changes in medium jejunum, proximal ileum and mesenteric lymph nodes	DON induced a decrease in villus height of jejunum, and a reduction in crypt depth of jejunum and ileum DON induced a decrease in number of mitotic figures, goblet cells in jejunum and ileum DON induced a decrease in number of lymphocytes in jejunum DON induced significant increase in lesional score and caspase-3 positive cells in lymph nodes	[30]

Materials and Methods

Animals

This study was conducted with twenty 4-weeks-old piglets (Dutch Landrace) obtained from commercial pig farm (weaning age: 3 weeks old). Animals were housed under conventional conditions at the experimental farm for pig production, Test and Training Center for Agriculture (Proef-en Vormingscentrum voor de Landbouw; PVL) in Bocholt, Belgium, and temperature and lightening programs were followed according to standard recommendations. The piglets were acclimatized for 2 weeks in the experimental facility and allocated to two groups (10 piglets per group) with an equal distribution of sex (4 male/6 female). The groups were housed in separate pens (10 piglets per pen) with free access to feed and water. Piglets were handled according to the Federation of European Laboratory Animal Science Associations FELASA guidelines and the Belgian law on the protection of animals, and the experimental protocol was agreed by the Ethical Committee on the use of experimental animals.

Experimental design

Pigs (6 weeks old) were given a commercial standard diet for piglets (Cibus NV, Poperinge, Belgium, Supplementary Table 1), to which DON was added. DON was incorporated into the diet at 0.9 mg/kg, added as the purified toxin (Romer Laboratories, Inc., Union, MO, USA).

The DON content of the diet used in our experiment was analyzed by HPLC analyses, and no DON contamination (detection limit: 10 µg/kg) was observed in the feed samples of the control diet.

The control and contaminated diets were fed for 10 days. The body weight (BW) of the piglets was recorded at the start and the end of the experiment, and the individual daily weight gain of the piglets was calculated based on the start and end weights for each individual animal. The start weights of the piglets were not significantly different (Table 3). Additionally, the relative weight gain was calculated by ((end weight-start weight)/start weight) × 100% per individual animal. The amount of feed consumed per group was determined, and the feed conversion ratio was calculated based on the feed intake measured per pen at the beginning and end of the trial. At the final day of the experiment, the animals received a single DON bolus via bottle feeding at a dose of 0.28 mg/kg BW (approximately 10-times higher than the daily DON dose in mg/kg BW) to evaluate the degree of oral absorption. Exactly two hours after the DON bolus, the piglets were sedated with an intramuscular injection of azaperone (Stresnil® Elanco Animal Health, Greenfield, IN, USA) (4 mg/kg) followed by induction of euthanasia via an intravenous injection of 200 mg/kg pentobarbital (Euthasol®, Virbac Animal Health, Carros Cedex, France). The entire gastrointestinal tract was removed, and after cutting the mesentery, the small and large intestine were aligned on a table. The small intestine was divided into three parts of equal length, and 10-cm tissue segments were collected

from the middle of each part, 10-cm segments were collected from the middle part of the caecum, and a 10-cm segment of proximal colon collected 30 cm distal to the ileocecal valve [31]. Digesta was removed from these segments by flushing with cold saline.

DON levels in plasma

DON levels in blood plasma were measured by standard high-performance liquid chromatography (HPLC) analyses with affinity column cleanup based on the method described in Janes and Schuster [32].

Histomorphometric analysis of the small intestine

The small intestine parts (duodenum and jejunum) were fixed in 10% neutral buffered formalin, embedded in paraffin and 5 µm sections were cut and stained with haematoxylin and eosin (H&E), according to standard methods. Photomicrographs were taken with an Olympus BX50 microscope (Olympus Europa GmbH, Hamburg, Germany) equipped with a Leica DFC 320 digital camera (Leica Microsystems, Wetzlar, Germany). The morphometric analysis of the sections was performed on 10 randomly-selected, well-oriented villi and crypts per animal. A computerized microscope-based image analyzer (Cell^D version 3.3; Olympus Europa GmbH, Hamburg, Germany, 2009) was used to determine histomorphometric parameters: villus height (measured from the tip of the villus to the villus-crypt junction), crypt depth (measured from the crypt villus junction to the base of the crypt), villus surface area (total surface of the villus), epithelial cell area (villus surface area minus villus area without epithelial cells), villus width, villus breadth top and villus breadth base. These regions of interest were manually defined for each villus separately.

Determination of mRNA expression in intestinal samples by qRT-PCR

For mRNA studies, the intestinal tissue segments (approximately 2-3 cm) were snap frozen in liquid nitrogen and stored at -80˚C for RNA isolation. Fifty milligrams of each sample were suspended in 350 µl RNA lysis buffer with β-mercaptoethanol and homogenized using a TissueLyser (Qiagen, Hilden, Germany) for 1 minute/25 Hz. RNA isolation was performed using spin columns according to the manufacturer's instructions (Promega, Madison, WI, USA). Subsequently, 1 µg of extracted total RNA was reverse transcribed with the iScriptTM cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). After reverse transcription, qRT-PCR was performed using a reaction mixture, containing 10 µl of the diluted cDNA mixed with 12.5 µl iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), forward and reverse primers (final concentration of 300 nM for each primer), and sterile deionized water, prepared according to the manufacturer's instructions. The MyIQ single-color real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) was used with MyIQ System 1.0.410 software (Bio-Rad Laboratories, Hercules, CA, USA, 2008). Gene specific primers for claudin1

(CLDN1), CLDN2, CLDN3, CLDN4, CLDN5, occludin (OCLN), zona occludens protein-1 (ZO-1), ZO-2, caspase-3, Ki67, glycoprotein (P-gp, ATP-binding cassette, sub-family B member 1 (ABCB1)), hypoxia-inducible factor 1-alpha (HIF-1α), heme-oxygenase1 (HMOX1), heme-oxygenase2 (HMOX2), cyclooxygenase-1 (COX-1), COX-2, interleukin-1 beta (IL-1β), interleukin-10 (IL-10) and Toll-like receptor 4 (TLR4) (Table 2) were derived from the NCBI GenBank and were manufactured commercially (Eurogentec, Seraing, Belgium). The specificity and efficiency of selected primers (Table 2) were confirmed by qRT-PCR analysis of dilution series of pooled cDNA at a temperature gradient (55°C - 65°C) for primer-annealing and subsequent melting curve analysis. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as reference gene, since HPRT1 is a valid reference gene for transcripts in different pig tissues [33].

Western blot analysis

Approximately 50 mg of each intestinal sample were lysed using 500 µl RIPA lysis buffer (Thermo scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany) and the total protein concentration was measured by the BCA protein assay kit (Thermo scientific, Rockford, IL, USA). Standardized protein amounts of boiled samples were isolated by electrophoresis (CriterionTM Gel, 4%-20% Tris-HCL, Bio-Rad Laboratories, Hercules, CA, USA) and electro-transferred onto polyvinylidene difluoride membranes (Bio-Rad, Veenendaal, The Netherlands). 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 anti-occludin (1:250, Abcam, Cambridge, UK). After washing in PBST, the membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (1:5000, Dako, Glostrup, Denmark) for 2 h 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 Laboratories, Hercules, CA, USA). In the next step the membranes re-probed with a β-Actin antibody (1:4000, Cell Signaling, Danvers, MA, USA) to assess the equality of loading. Signal intensities were quantified using the ImageJ 1.47 software (National institutes of Health, Bethesda, MD, USA, 2013) and the protein expression was normalized with β-Actin and expressed as the mean fold change in relation to the control group.

Immunohistochemistry

After fixation in 10% formalin, jejunum samples were embedded in paraffin. Paraffin sections (5 µm) were deparaffinized, and endogenous peroxidase activity was blocked with 0.3% H_2O_2 (Merck, Darmstadt, Germany) in methanol (30 minutes) and rehydrated in a graded ethanol series to PBS. After antigen retrieval in 10 mM citrate buffer (PH 6.0) for 10 minutes in a microwave, sections were pre-treated with 5% goat serum (Dako, Glostrup, Denmark) before overnight incubation with rabbit-polyclonal Ki67

antibody (1:1000, ab66155, Abcam, Cambridge, UK) or caspase-3 (1:1000, Cell Signaling, Danvers, MA, USA) at 4˚C. Tissue sections were sequentially incubated with biotinylated goat-anti rabbit (1:200, Dako, Glostrup, Denmark) followed by streptavidin-biotin complex/horseradish peroxidase (Vectastain Elite ABC, Vector Laboratories, Peterborough, UK). Staining was visualized using 0.05% diaminobenzidene (DAB) solution for 10 minutes, and sections were counterstained with Mayers' haematoxylin (Merck Millipore, Amsterdam, The Netherlands). Photomicrographs were taken with an Olympus BX50 microscope (Olympus Europa GmbH, Hamburg, Germany) equipped with a Leica DFC 320 digital camera (Leica Microsystems, Wetzlar, Germany).

Table 2. Primer sequences of genes used for qRT-PCR analysis.

	Primer sequence (5'-3')			
Target genes	**Forwrad**	**Reverse**	**AT**	**References**
ABCB1	TGGCAGTGGGACAGGTTAGTTC	CACGGTGCTTGAGCTGTCAATC	65	AY825267
Caspase-3	AGAGGGGACTGCTGTAGAACT	CCGTCTCAATCCCACAGTCC	58.7	NM_214131.1
CLDN1	TGGCTCCGCGTCTCAGTCC	TGCGAGGGGTGCAGGTCTAA	65	NM_001244539.1
CLDN2	CTCGTTGGCCTGTATCATCACC	CAGGGGGGAGTAGAAGTCCC	63.1	NM_001161638.1
CLDN3	AACACCATCATCCGGGACTTC	CGCGGAGTAGAGGATCTTGG	61.2	NM_001160075.1
CLDN4	AGGAGAGACGCTTCAATCGG	GTCCAGACACCTGAACACCG	63.1	NM_001161637.1
CLDN5	CTCTGCTGGTTCGCCAACA	CAGCTCGTACTTCTGCGACATG	58.7	NM_001161636.1
COX-1	CAAGATGGGTCCTGGCTTCA	CCATAAATGTGGCCGAGGTCTA	64.3	XM_001926129.4
COX-2	CATTGATGCCATGGAGCTGTA	CTCCCCAAAGATGGCATCTG	64.3	NM_214321.1
HIF-1α	GCTTGCTCATCAGTTGCC	GCCTTCATTTCATCTTCAATATCC	64.3	AY485675.1
HMOX1	AGACCGCCTTCCTGCTCA	GGGTCTCTGGTCCTTAGTGTC	64	NM_001004027
HMOX2	GCAGCAGTTCAAGCAGTTCT	CCTCCTCCACGATCTTCTCT	63.1	NM_001244412.1
HPRT	CTGAACGGCTTGCTCGAGAT	TCCAGCAGGTCAGCAAAGAA	63.1	NM_001032376.2
IL-10	CGGCGCTGTCATCAATTTCTG	CCCCTCTTGGAGCTTGCTA	58.7	NM_214041
IL-1β	GTGCAAACTCCAGGACAAAGACCA	CACAAGCTCATGCAGAACACCAC	61.2	NM_214055
Ki67	TCTTGTCCCTGAATCCGCAA	TGTTTCTCTGGTTGCTTGGTTG	61.2	NM_001101827.1
OCLN	ATCAACAAAGGCAACTCT	GCAGCAGCCATGTACTCT	55.8	NM_001163647.2
TLR4	CAAGGACCAGAAGCAGCTCC	GACGGCCTCGCTTATCTGAC	63.1	AB188301.2
ZO-1	GAGTTTGATAGTGGCGTT	GTGGGAGGATGCTGTTGT	58.7	XM_005659811.1
ZO-2	GCAGAGACAACCCCCACTTT	CGTTAACCATGACCACCCGA	55.8	NM_001206404.1

AT, annealing temperature (°C)

Statistical analysis

Experimental results are expressed as the mean ± SEM. Data were analyzed with univariate analysis of variance (ANOVA) using SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL, USA, 2013). Analysis was performed on data in a 2 × 2 (diet × sex) between-subject factorial design. Results were considered statistically significant when $P < 0.05$. A gender effect was only observed in the relative OCLN expression in caecum ($P = 0.015$). Differences in feed intake and food conversion ratio between groups were not statistically determined, since these data are based on total feed intake per experimental group.

Results

Average daily gain is decreased by 0.9 ppm DON in the diet

No alterations were observed in the general health conditions of the piglets during the experimental period. However, the growth performance of piglets fed the DON diet was affected, since these piglets showed a significantly lower relative weight gain, as well as a lower average daily gain (kg/day) compared to control piglets. There was no obvious difference between the total feed intake of the group piglets fed a DON diet compared to the group fed a control diet. However, a higher feed conversion ratio was observed in the group piglets fed the DON diet (Table 3).

Table 3. Body weight (BW), relative weight gain, average daily gain, feed intake and feed conversion ratio.

Item	Start weight (kg)		End weight (kg)		Relative weight gain (% increase)		Average daily gain (kg/day)		Feed intake (kg/day)	Feed conversion ratio
Exp. group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	Mean
Control	8.67	0.48	10.98	0.53	27.21	1.82	0.29	0.01	0.31	1.12
DON	7.87	0.47	9.48	0.61	**20.17***	1.15	**0.20*****	0.01	0.30	1.57

*P < 0.05; ***P <0.001; relative weight gain = ((end weight-start weight)/start weight) × 100% per individual animal.

Detectable DON in plasma levels after bolus administration

The average values of plasma DON in the piglets sampled at 2 h after receiving a DON bolus (0.28 mg/kg BW) and fed a DON diet for 10 days were 168 ± 16.1 ng/ml. DON plasma levels were below the detection limit in the control group (detection limit: 0.05 µg/ml).

Low DON levels can induce histomorphological changes in the piglet intestine

Providing a DON-contaminated diet resulted in a decreased villus height (Figure 1A and 2A), epithelial cell area (Figure 1C and 2C) and area without epithelial layer (Figure 1D and 2D) in both duodenum and jejunum. The crypt depth was significantly increased in the jejunum of DON-treated piglets compared to piglets fed a control diet (Figure 2B). Representative photomicrographs of villi in duodenum (Figure 1E and 1F) and jejunum (Figure 2E and 2F) from control (Figure 1E and 2E) and DON-fed piglets (Figure 1F and 2F) are depicted in Figures 1 and 2 and showed that villi in both duodenum and jejunum of piglets given the DON diet for 10 days were proportionally smaller compared to the villi from control animals. The ratio between the epithelial cell area and villus area without epithelial cell area remained the same in DON-fed animals compared to the control animals (Supplementary Figure 1). Additionally, in the duodenum, a slight, but significant decrease in the villus breadth top is observed in the pigs receiving a DON

diet, while in the jejunum, the villus breadth base is significantly decreased in the DON-fed pigs compared to control pigs (Supplementary Figure 1).

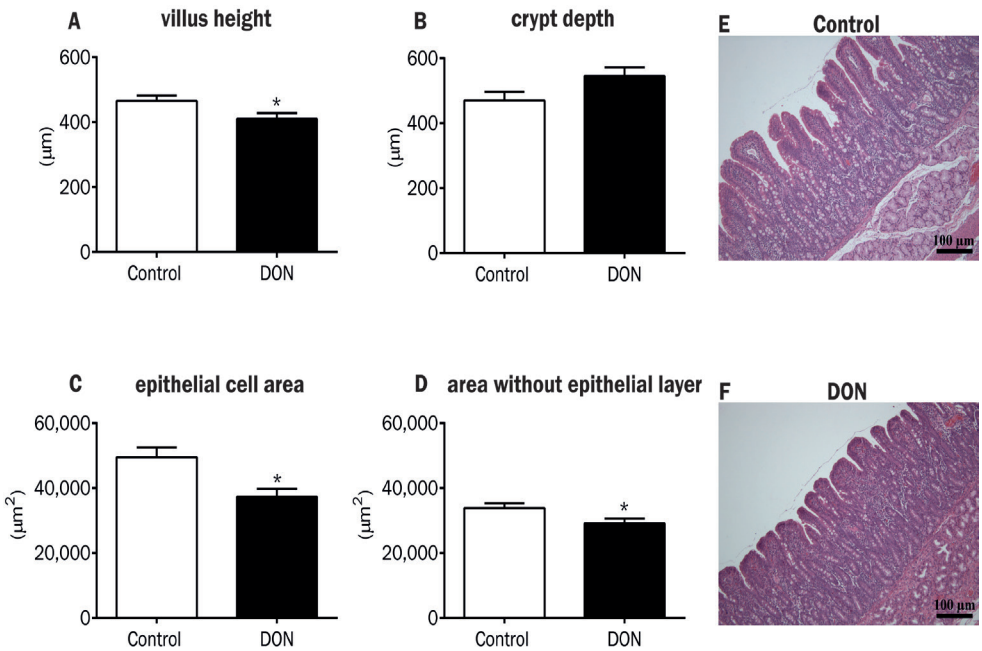

Figure 1. DON induces histomorphological changes in the piglet duodenum even at low exposure levels. Piglets were fed a control or DON diet (0.9 mg/kg feed) for 10 days; two hours after the DON bolus (0.28 mg/kg BW), segments obtained from duodenum were fixed in 10% formalin, and paraffin sections were H&E-stained for histomorphometric analysis of villus height (A), crypt depth (B), epithelial cell area (C) and area without epithelial layer (D); representative photomicrographs of villi in duodenum (Figure 1E and 1F) from control (E) and DON-fed piglets (F). Magnification: 100x. Results are expressed as means ± SEM; n = 9–10 animals/group. *$P \leq 0.05$; significantly different from the control group.

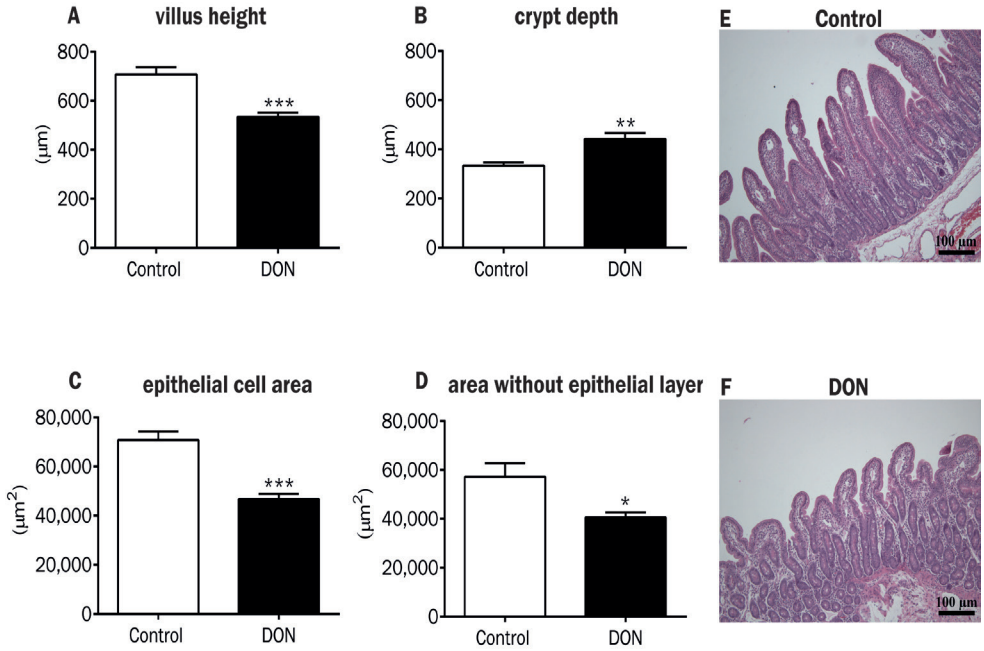

Figure 2. DON induces histomorphological changes in the piglet jejunum even at low exposure levels. Piglets were fed a control or DON diet (0.9 mg/kg feed) for 10 days; two hours after the DON bolus (0.28 mg/kg BW), segments obtained from jejunum were fixed in 10% formalin, and paraffin sections were H&E-stained for histomorphometric analysis of villus height (A), crypt depth (B), epithelial cell area (C) and area without epithelial layer (D); representative photomicrographs of villi in jejunum from control (E) and DON-fed piglets (F). Magnification: 100x. Results are expressed as means ± SEM; n = 9–10 animals/group. *P < 0.05, **P < 0.01, ***P < 0.001; significantly different from the control group.

Several markers for barrier integrity, inflammation and oxidative stress in the intestines are affected by the DON diet

Tight junction proteins

qRT-PCR analysis was performed to investigate DON-induced alterations in barrier integrity by measuring the mRNA expression levels of various tight junction (TJ) proteins, including CLDN1-5, OCLN, ZO-1 and ZO-2 in different parts of the intestine (duodenum, jejunum, ileum, caecum and colon). The mRNA expression of ZO-1 and OCLN was upregulated in duodenum of piglets fed a DON diet compared to piglets fed a control diet (Figure 3, duodenum). Gene expression levels of CLDN4, OCLN, ZO-1 and ZO-2 in jejunum of DON-treated piglets were clearly downregulated compared to control animals (Figure 3, Jejunum). In the ileum of piglets fed the DON diet, especially the mRNA

expression of OCLN was upregulated compared to the control animals (Figure 3, ileum). Increased mRNA expression levels of CLDN1, CLDN3, CLDN4, CLDN5 and OCLN were observed in the caecum of piglets fed a DON diet compared to the control diet (Figure 3, caecum). An increase in mRNA levels of OCLN and ZO-2 related to the DON diet was found in the colon of piglets fed the DON-contaminated diet (Figure 3, colon).

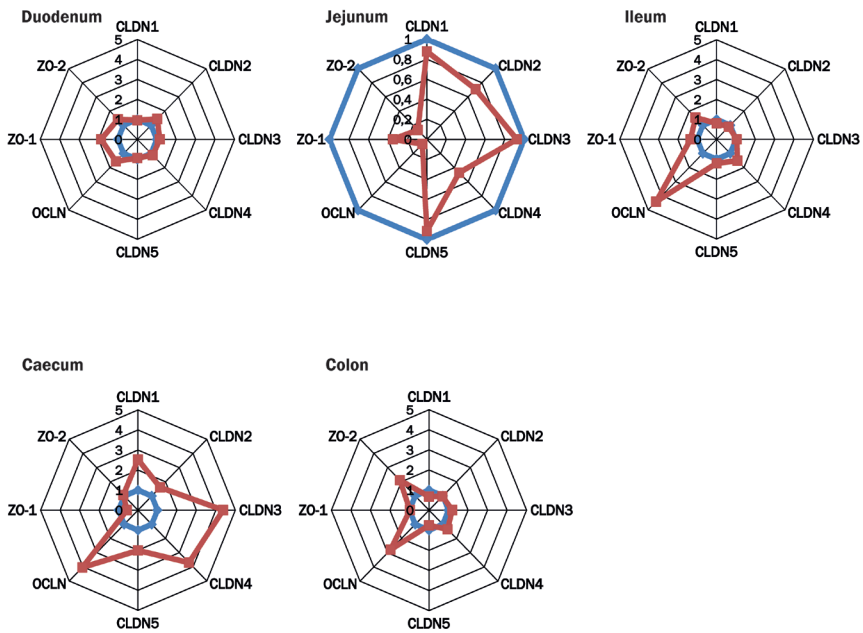

Figure 3. The mRNA expression levels of markers of barrier integrity are affected by short-term, low-dose exposure to DON. Piglets received a control (blue line) or DON diet (0.9 mg/kg feed, red line) for 10 days; two hours after the DON bolus, samples from different parts of the intestine (duodenum, jejunum, Ileum, caecum and colon) were collected, and mRNA levels of various TJ proteins (CLDN1, 2, 3, 4, 5, ZO-1, ZO-2 and OCLN) were measured by qRT-PCR. Results are expressed as the relative mRNA expression as means ± SEM; n = 9–10 animals/group.

Additionally, low-dose DON exposure also affected the protein expression of OCLN in the different parts of the intestine. As illustrated in Figure 4, the protein expression of OCLN is significantly increased in duodenum, jejunum and colon of DON-treated animals compared to control animals.

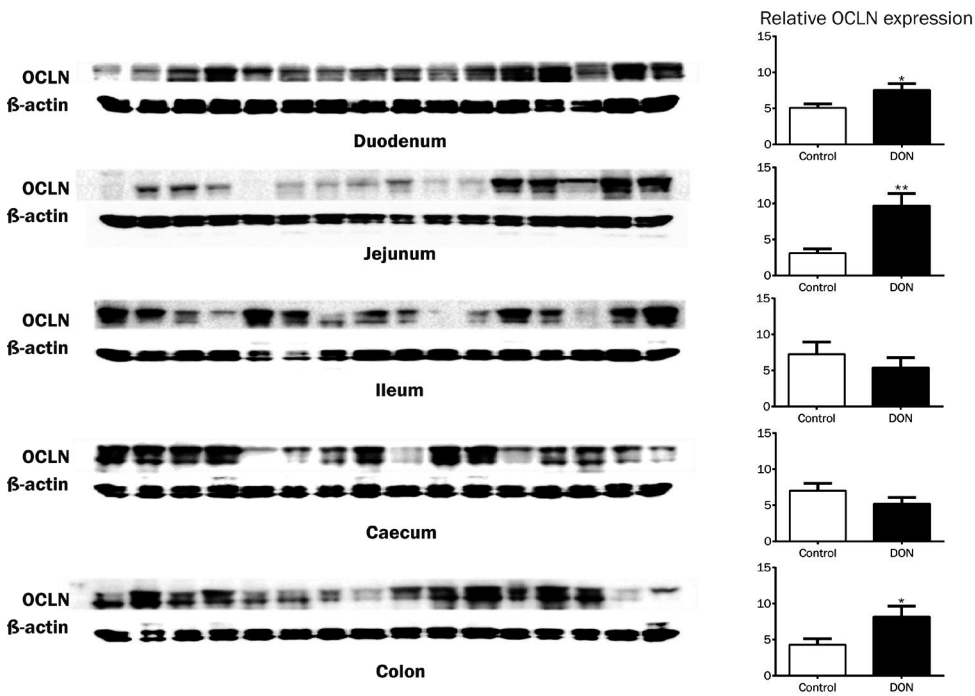

Figure 4. The protein expressions of occludin in different parts of the intestine are affected by short-term, low-dose exposure to DON. Piglets received a control diet or DON diet (0.9 mg/kg feed) for 10 days; two hours after the DON bolus, samples from different parts of the intestine (duodenum, jejunum, Ileum, caecum and colon) were collected, and protein levels were measured by Western blot analysis. Results are expressed as the relative protein expression (normalized to β-actin) as means ± SEM; n = 8 animals/group. *P < 0.05, **P < 0.01; significantly different from the control group.

Inflammatory markers

The effect of DON on the development of inflammation was investigated by measuring the mRNA expression levels of COX-1, COX-2, IL-1β, IL-10 and TLR4 in the intestine. IL-10 and IL-1β mRNA levels were increased in the duodenum of piglets fed a DON diet compared to a control diet (Figure 5, duodenum), while they were slightly decreased by DON in the jejunum (Figure 5, jejunum). Enhanced COX-2 mRNA levels were found in caecum of DON-treated piglets compared to control piglets (Figure 5, caecum). COX-1 and TLR4 remained unchanged after DON treatment.

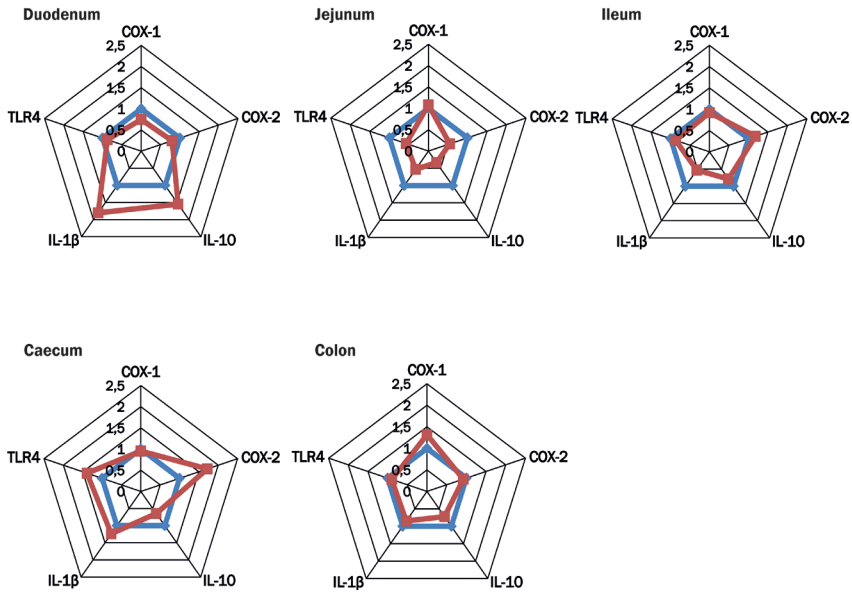

Figure 5. The mRNA expression levels of markers of inflammation are affected by short-term, low-dose exposure to DON. Piglets received a control (blue line) or DON diet (0.9 mg/kg feed, red line) for 10 days; two hours after the DON bolus, samples from different parts of the intestine (duodenum, jejunum, Ileum, caecum and colon) were collected, and mRNA levels of inflammatory markers (COX-1, COX-2, IL-10, IL-1β and TLR4) were measured by qRT-PCR. Results are expressed as the relative mRNA expression as means ± SEM; n = 9–10 animals/group.

Oxidative stress markers

In addition to the specific markers of inflammation, the mRNA levels of HIF-1α, HMOX1 and HMOX2 were measured in the intestine as potential markers of oxidative stress. HMOX1 was downregulated in the jejunum after exposure to a DON-contaminated diet (Figure 6, jejunum), while especially in the colon of piglets fed the DON diet, increased HMOX1 levels were identified as compared to piglets fed the control diet (Figure 6, colon).

Efflux transporter

As DON is a substrate for efflux transporters, the permeability glycoprotein ABCB1 mRNA expression levels were assessed in different parts of the intestine. Downregulated mRNA expression levels of ABCB1 were only detected in the jejunum after DON exposure (Figure 6, jejunum).

Proliferation and apoptosis

The mRNA levels of Ki67, a proliferative marker, and caspase-3, a marker for apoptosis, were downregulated in jejunum of piglets fed the DON diet compared with piglets fed the control diet (Figure 6, jejunum).

No remarkable effects were observed in the other parts of the intestine (Figure 6, duodenum, ileum, caecum and colon). No obvious changes were observed in the immuno-histochemical staining for Ki67 and caspase-3 between the jejunum of piglets fed the DON diet and piglets fed the control diet (Figure 7).

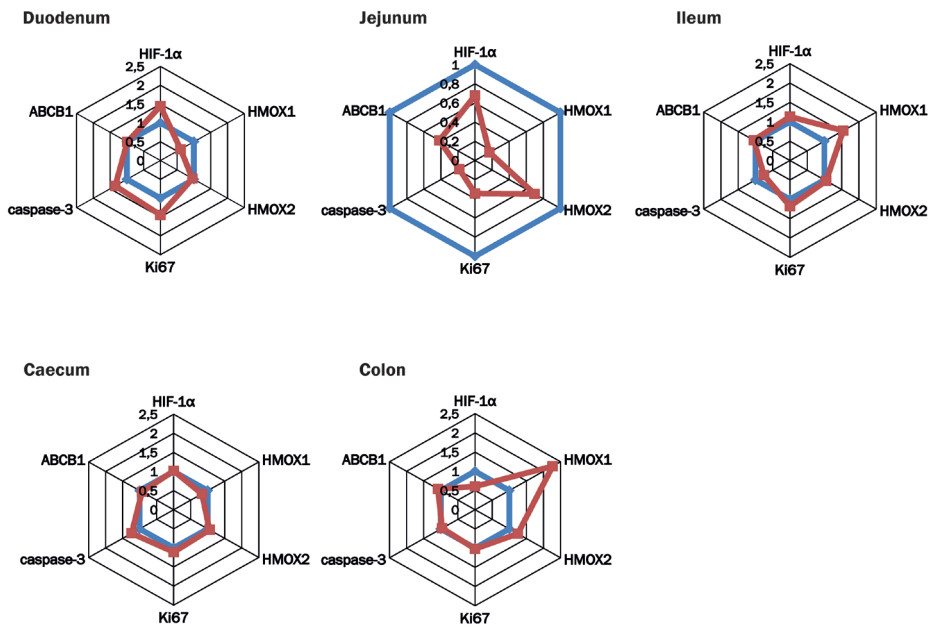

Figure 6. The mRNA expression levels of markers of oxidative stress, efflux transporter, proliferation and apoptosis are affected by short-term, low-dose exposure to DON. Piglets received a control (blue line) or DON diet (0.9 mg/kg feed, red line) for 10 days; two hours after the DON bolus, samples from different parts of the intestine (duodenum, jejunum, Ileum, caecum and colon) were collected, and mRNA levels of stress markers (HIF-1α, HMOX1, HMOX2), an apoptotic and a proliferative marker (caspase-3 and Ki67) and an efflux transporter (ABCB1) were measured by qRT-PCR. Results are expressed as the relative mRNA expression as means ± SEM; n = 9–10 animals/group.

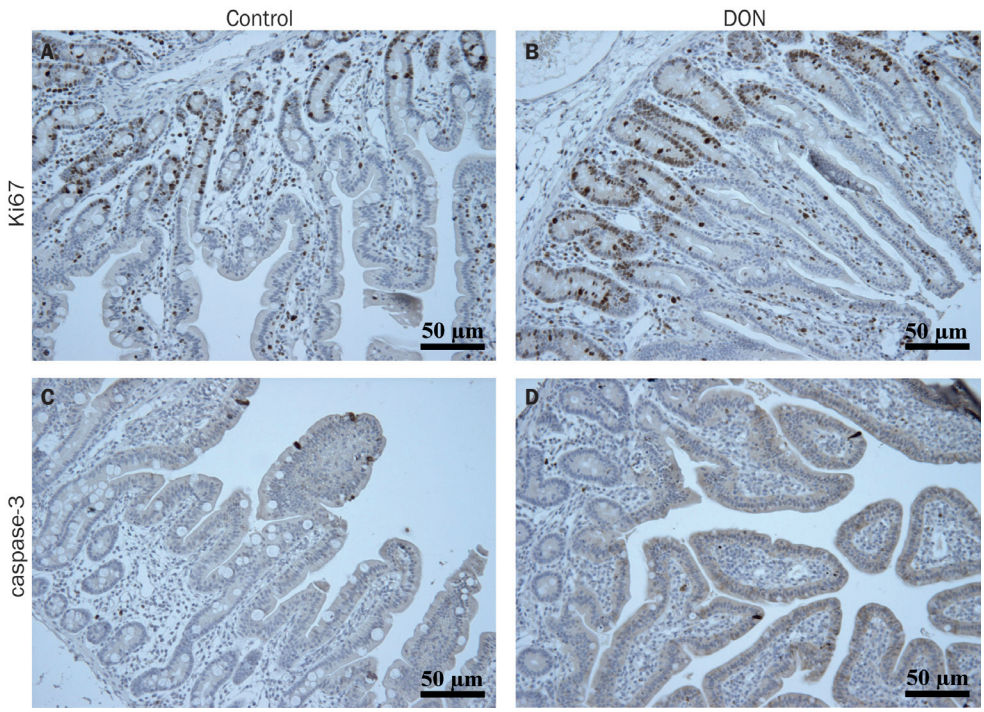

Figure 7. No obvious changes in the expression of proliferation marker Ki67 and apoptotic marker caspase-3 between jejunum of control and DON-treated piglets. For immunohistochemical staining, Swiss-rolled paraffin sections obtained from the jejunum of control (Figure 7A and 7C) and DON-treated (Figure 7B and 7D) piglets were detected by antibodies for Ki67 or caspase-3, as described in the Materials and Methods. Representative images are shown (n = 5 piglets/group). Magnification: 200x.

Discussion

The aim of this study was to identify biomarkers related to gut health that might be indicative for changes at low exposure level, hence support risk characterization of DON in pigs and the assessment of intervention strategies. Growing pigs were given low DON levels (0.9 mg/kg feed) in feed for 10 days, which is the recommended maximal concentration at which no signs of clinical intoxications are expected. Conversion of the concentration in feed (i.e. 0.9 ppm) and the daily feed intake per kg/BW, this in-feed application corresponded to a dose varying between 0.04 mg/kg BW at the beginning to 0.03 mg/kg BW at the end of the 10 days exposure period.

The most common clinical signs due to prolonged dietary exposure to DON in different experimental animal models are depression in feed intake and weight gain [11,34–36]. Different studies reported that pigs could tolerate up to 0.6-0.9 mg of dietary DON/kg feed without any adverse effects on feed consumption and BW gain (European Food Safety Authority, 2004 table 3) [1]. In the present study, the average (relative) daily weight gain was negatively affected by the given dose of DON and subsequently an enhanced feed conversion ratio was calculated, although this ratio was not significant different. The feed intake of piglets fed a low dose DON diet remained the same as piglets fed a control diet, which is also observed in other studies [37,38]. This significant effect on the average daily gain by this low contamination level of DON in diet together with a short exposure period of 10 days was not expected. However, an old study conducted with naturally contaminated grains showed a temporary reduction on feed intake even in pigs given a low DON dose in the diet (below 1 mg DON/kg feed) [39].

In the current study, the average DON levels in plasma of piglets fed a DON diet for 10 days sampled at 2 h after receiving a DON bolus (0.28 mg/kg BW) were 168 ± 16.1 ng/ml. This maximum absorption rate was determined as a reference point for further studies with dietary components that may impair the absorption of DON. In a mouse study, DON levels observed in plasma 120 minutes following acute oral DON exposure (5 mg/kg BW) were 260 ng/ml [40]. Comparable data in pigs are lacking. However, in chronic exposure studies with DON in pigs, much lower DON levels in plasma were observed, since data by these studies showed DON levels in blood of 5-17 (10.5) ng/ml after a chronic DON diet (2.3 mg/kg feed) for 28 days [41] and 7-30 (22.5) ng/ml after a DON diet (4.5 mg/kg feed) for five weeks [34]. In one of the recently published studies, DON plasma levels were 13.2 ng/ml after a DON diet (3.1 mg/kg feed) for 37 days [42].

Intestinal epithelial cells are considered to be one of the primary targets following dietary DON exposure, since DON mainly enters the body via the oral route [43]. The most prominent alterations in the current investigations concerned villus flattening along the intestine with a related decrease in epithelial cell area and the area without epithelial layer (mucosal area) in both duodenum and jejunum of DON-exposed animals, while the intestinal epithelial layer was not disrupted. Alternatively to the assumption that the morphological changes are related to the direct effects of the epithelial cells,

Blikslager *et al.* [44] discussed that the decreased villus height could be explained by villus contraction, which aids the restoration of the barrier function via reducing the surface area. This decrease in villus height and corresponding reduced surface area in the intestines may impair nutrient transport and utilization, which could at least partly explain the decrease in average daily gain in the DON-fed animals. The ability of DON to decrease nutrient absorption has been previously reported ([17,45,46] and reviewed in [3]). Various other studies also observed a smaller villus height in jejunum after dietary DON exposure, albeit at higher exposure levels (Bracarense *et al.* [16] DON 3 mg/kg feed for 5 weeks; Lucioli *et al.* [21] DON 2.3 mg/kg feed for 35 days; Pinton *et al.* [25] DON 2.3 mg/kg feed for 4 weeks).

In contrast to the decrease in villus height after DON exposure, the crypt depth is increased in the jejunum of piglets exposed to DON in the current study. This is consistent with observations of Dänicke *et al.* [24], who found DON-related effects on crypt depth in the jejunum (3.1 mg DON/kg feed for 10 weeks). It is believed that deeper crypts are an indication of a high cell turnover to permit the renewal of the villus in response to inflammation and toxic damage [47,48]. In light of this assumption, we investigated whether DON exposure indeed affected the proliferation rate of intestinal cells, and subsequently, the proliferative marker Ki67 was evaluated. Down-regulated mRNA levels of Ki67 in the jejunum of DON-fed pigs were found, while no obvious changes in proliferating and apoptotic cells were observed in the immunohistochemistry staining for Ki67 and caspase-3 in the jejunum of DON-fed piglets compared to control piglets. This would suggest that at this low level, DON does not clearly influence the cell turnover in the intestines.

The intestinal barrier, the first target for toxins, like DON, is composed of epithelial cells connected by an intercellular junctional complex consisting of tight junctions, adherens junctions, gap junctions and desmosomes, among which the TJs represent the major determinants of the paracellular permeability. The TJ protein OCLN is an important integral membrane TJ protein involved in TJ stabilization and formation of a highly effective barrier [49]. Results of the present study show that especially the tight junction protein OCLN was upregulated by DON exposure in different parts of intestine (duodenum, ileum, caecum and colon). This effect, an upregulation of OCLN after DON exposure, is also reported in *in vitro* studies with Caco-2 cells (4.17 μM DON for 3 h, 6 h and 24 h [6], 5000 ng/ml for 24 h [50]). *In vivo* studies related to chronic DON exposure in piglets mainly focus on the effect on TJ protein expression in the intestine without any investigation on gene expression levels [16,26]. Here, it has been illustrated that the protein expression of OCLN in duodenum, jejunum and colon was significantly increased after low-dose DON exposure compared to control pigs, which is in line with the increase in OCLN mRNA expression in duodenum and colon. Surprisingly, there is a discrepancy between the decrease in mRNA expression of OCLN in the jejunum of piglets fed a DON diet and the increase in OCLN protein expression in the jejunum after a DON diet. There is no clear explanation for these findings, but we can speculate that this could be related

to the short-term, low-dose exposure to DON. A higher DON dose (3 mg/kg feed) for a longer period (five weeks) resulted in a reduced OCLN protein expression in the pig ileum [16]. An *in vitro* study by Gu *et al.* [51] also showed a decrease in OCLN protein expression in IPEC-J2 cells after DON exposure (2µg/ml) for 48 h.

Akbari *et al.* [6] described that in mice, the main effect on the TJ network exerted by a high acute DON dose (25 mg/kg BW) is the modulation of the CLDN expression. In the current experiment, an up-regulation of claudins, CLDN1, 3 and 4, was observed in the caecum of DON-fed piglets, while in the jejunum the mRNA expression of different TJ proteins (OCLN, ZO-1 and ZO-2) is downregulated. Recently, Lessard *et al.* [52] also showed a down-regulation in mRNA expression of CLDN3, 4 and OCLN in ileum of piglets fed a DON diet (3.5 mg/kg feed) for 42 days compared to controls.

The clinical relevance of these various alterations in tight junction protein expression along the intestine cannot be clearly explained, but predict a difference in the TJ expression pattern and the susceptibility of the different parts of the intestine against DON. A comprehensive study in the mice intestine pointed out that the adult mouse intestine expresses many different claudins in complex quantitative and spatial patterns, and this pattern could also be affected by age [53]. Comparable studies in pigs are not available, but would be needed for further interpretations of the obtained results. Another explanation could be the difference in exposure side in different parts of the intestine, since DON absorption mainly occurred in the upper small intestine, while the large intestine is also exposed to DON via the blood stream [54]. This is reinforced by Diesing *et al.* [55] who showed that apical and basolateral exposure of DON to epithelial cell layers trigger different gene response profiles. Akbari *et al.* [6] also observed differences in the routes of DON exposure in an *in vitro* Caco-2 transwell system. Interestingly, Waśkiewicz *et al.* [29] showed that administration of low doses DON to piglets resulted time-dependently in increasing DON concentrations in the intestines, while the relative concentrations in the liver decreased. Within the intestinal tissue, the DON tissue concentrations varied in the different segments, with increasing concentrations over time in the ileum. These time-dependent changes could be a possible explanation for the differences in mRNA expression between the different intestinal segments.

A number of studies suggest that DON is a potent immuno-modulator acting as an immunosuppressive, as well as an immuno-stimulatory agent [56]. Most *in vivo* studies focus on a wide array of pro-inflammatory cytokines and chemokines that are detectable in different organs, like spleen, liver, kidney and lung, after DON exposure [57–59]. The mRNA expression of different cytokines and chemokines along the intestine after dietary DON is not yet abundantly investigated. In this study, both IL-1β and IL-10 mRNA levels were increased in the duodenum of piglets fed a DON diet compared to control piglets. Bracarense *et al.* [16] showed that in ileum or jejunum of piglets fed a DON diet (3 mg/kg feed) for five weeks the TNF-α, IL-1β, IFN-γ, IL-6 and IL-10 expression was significantly upregulated, while results of Lessard *et al.* [52] revealed that mRNA expression of IL-8, CXCL10 and IFN-γ was upregulated in ileum of pigs fed a DON

contaminated diet (3.5 mg DON/kg feed). In mice, it has been observed that DON at high doses of 25 mg/kg BW within 2 h transcriptionally upregulates the expression of IL-10 and IL-1β in Peyer's patches [60], while IL-1β, IL-6 and TNF-α mRNA levels in Peyer's patches were upregulated after short-term repeated oral exposure to DON (5 mg/kg BW) [57]. In contrast, Becker *et al.* [28] observed a downregulation for IL-1β and IL-8 in ileum from piglets after chronic exposure to low-level DON and discussed that the decreased expression of the cytokines is due to the indirect DON-mediated effect of reduced feed consumption. We observed a tendency towards downregulated cytokine levels in the jejunum of piglets fed a DON diet.

Furthermore, in the large intestines, where COX-2 is one of the most prominent markers of inflammation, a slight increase in COX-2 mRNA level was observed in the caecum of piglets fed a DON diet. This is comparable to different *in vitro* studies showing also an increase in COX-2 expression in intestinal epithelial cell lines [61,62], as well as in macrophages [56,63,64].

Cellular oxidative stress is one of the non-specific responses of cells to toxic or inflammatory injury. Oxidative stress can be the source of degradation in membrane phospholipids, which corresponds to a decrease in membrane transport, integrity and to a hyperpermeability of the intestinal barrier [65]. Studies with different cell lines indicated that DON has a capacity to induce oxidative stress [61,66,67]. One of the most sensitive indicators of oxidative stress is HMOX1, and in this study, the mRNA levels of HMOX1 were upregulated in the colon of the DON-fed piglets. Another recent piglet study described an effect of DON on other oxidative stress markers, since an increase in glutathione peroxidase 2 was observed, whereas expression of genes encoding enzymatic antioxidants and superoxide dismutase 3 were downregulated in pigs fed a DON diet (3.5 mg/kg feed) for 42 days [52]. Sheth *et al.* [68] and Basuroy *et al.* [69] described an effect of oxidative stress on the phosphorylation and redistribution of OCLN and ZO-1 in the TJ complex of the intestinal barrier, which could partly explain the observed changes in OCLN expression along the intestine.

Different studies reported the fact that DON is a substrate for ABC transporters influencing the rate of absorption in the upper part of intestine and facilitating secretion of circulating DON in lower parts [70,71]. It should be stressed that in our study the low doses of DON did not result in an alteration in ABCB1 mRNA levels in different parts of intestine, except a downregulation in the jejunum, which is in line with a previous study where DON (1 and 3 ppm) decreases intestinal ABCB1 expression within 10 days [72]. In our study, a low dose of DON was added to the DON diet, and at the end of the experiment, the animals received a DON bolus. It cannot entirely be excluded that the bolus application influenced some of the results, particularly regarding the PCR results in the upper intestines. However, almost no changes in mRNA expression in the duodenum were observed and only small differences in the histomorphology between duodenum and jejunum were measured.

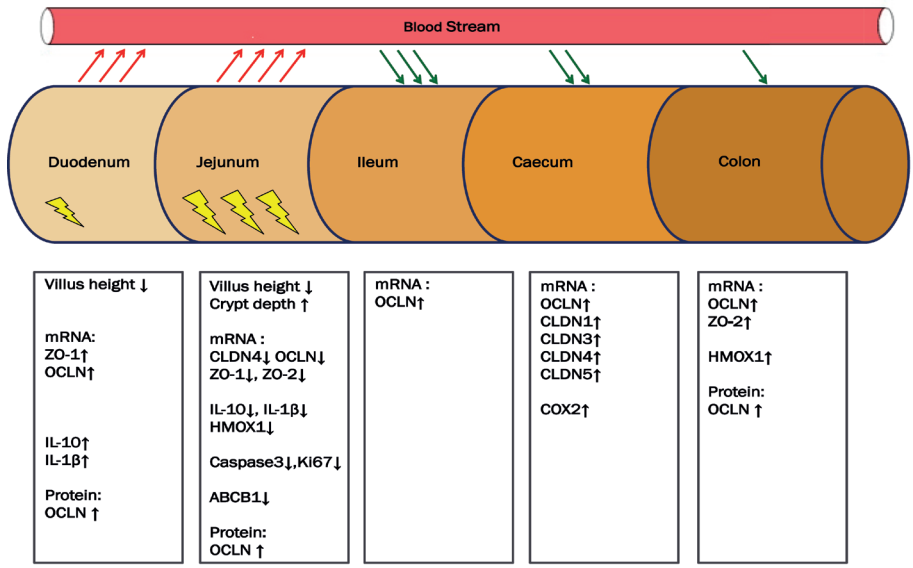

Figure 8. Schematic overview of DON-related effects in different segments of the intestine. Red arrows indicate absorption of DON. Green arrows indicate secretion of DON. The rectangles below each segment of the intestine summarize the impact of DON on villus architecture (duodenum and jejunum) and on mRNA expression levels of different genes related to intestinal barrier function (and OCLN protein expression), oxidative stress and inflammation. Negative effects of DON (⚡) are mainly observed in the jejunum (downregulation of different genes). Up arrows indicate increase and down arrows indicate decrease.

Conclusion

Taken together, the presented results show that low-level DON exposure (0.9 mg/kg feed) can even, after a short exposure period, impair weight gain accompanied by distinct changes in the intestinal tract of growing piglets, as depicted in Figure 8. Moreover, several indicators associated with intestinal barrier function, oxidative stress and inflammation, identified in previous studies as typical signs of long-term and high-dose exposure to DON, were already altered after the chosen short exposure period of 10 days. These results confirm that the intestines are an important target of DON toxicity, particularly at low dietary doses. This is not only of relevance for a further risk characterization of DON exposure to growing pigs, but might also provide the template for studies devoted to assessment of intervention strategies to mitigate adverse health effects of DON.

Acknowledgments
The authors kindly acknowledge the technical support by the staff of the Test and Training Center for Agriculture, Bocholt, and Sanluc International (Gijzenzele, Belgium) during the animal experiments.

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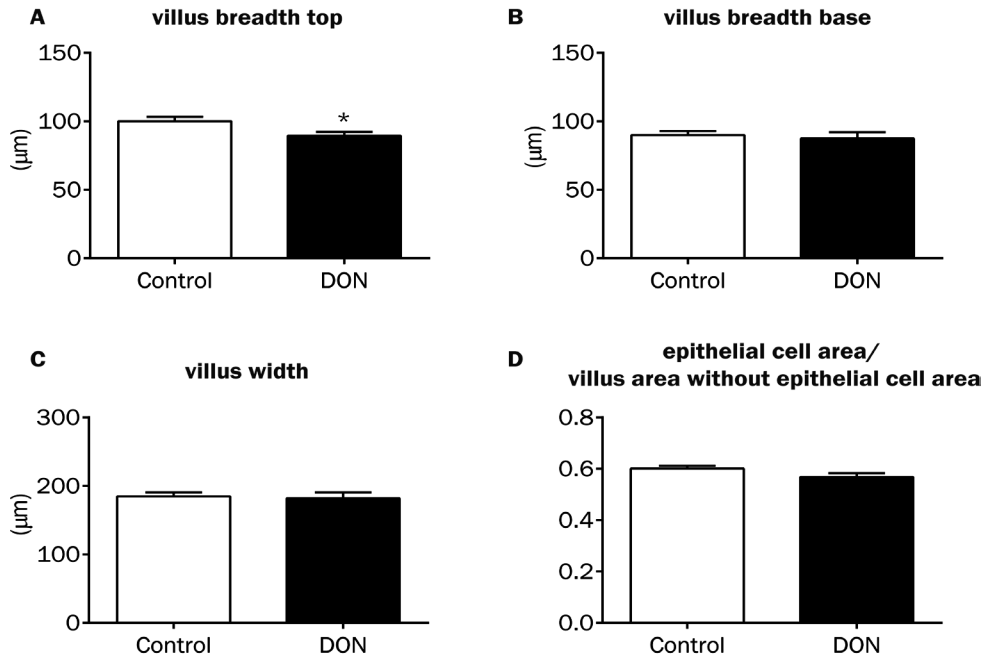
Supplementary data

Supplementary Table 1. Feed composition of standard piglet diet.

Ingredients	Value (g/kg)	Ingredients	Value (g/kg)
Wheat 2012	299.7	Wheat gluten (Roquette, Lestrem, France)	30
Barley 2012	250	Beet pulp sugar	20
Maize 2012	114	Fat + 88% triglyceride	8.5
Soybean (Danex, Izegem, Belgium)	110	Ultracid (INVE, Dendermonde, Belgium)	5
Soya 49/3.5	92.7	Threonine	0.1
Premix (Vimix, Deerlijk, Belgium)	70		
Nutrients	**Value**	**Nutrients**	**Value**
Crude protein (g)	171.30	6-phytase (FYT)	2,100.00
Lysine (g)	12.17	Sodium (g)	1.47
Digestible lysine (g)	10.57	Chlorine (g)	3.10
Methionine (g)	4.39	Potassium (g)	7.83
Digestible methionine (g)	3.96	Sodium + potassium-chloride (meq)	192.13
Digestible cystine (g)	2.24	Magnesium (g)	1.57
Digestible methionine + cystine (g)	6.26	Vitamin A per kg (IE)	15,050.00
Digestible threonine (g)	6.55	Vitamin D3 per kg (IE)	2,030.00
Digestible tryptophan (g)	2.00	Vitamin E per kg (IE)	120.33
Sugar + starch (g)	454.83	Butylated hydroxytoluene (ppm)	150.15
Sugar (g)	35.60	Formic acid (ppm)	1,633.00
Crude starch (g)	401.50	Lactic acid (ppm)	2,105.00
Crude fiber (g)	35.96	Moisture (g)	115.86
Net energy (kCal)	2,347.17	Weight (%)	100.00
Crude fat (g)	47.98	Cu premix copper sulfate (ppm)	148.03
Linoleic acid (g)	18.98	Fe premix sulfate (ppm)	198.17
n-3 poly unsaturated fatty acid (g)	2.26	Mn premix (oxide) (ppm)	52.22
n-6 poly unsaturated fatty acid (g)	18.70	Zn premix (sulfate) (ppm)	101.85
Crude ash (g)	49.81	I premix (calcium iodate) (ppm)	1.12
Ca (g)	7.09	Co carbonate (ppm)	1.12
Phosphorus (g)	5.12	Se sodium selenite (ppm)	0.29
Digestible phosphorus pellet (g)	3.50	Bacterial endo-xylanase (IU)	11.06
Digestible phosphorus flour (g)	3.78		

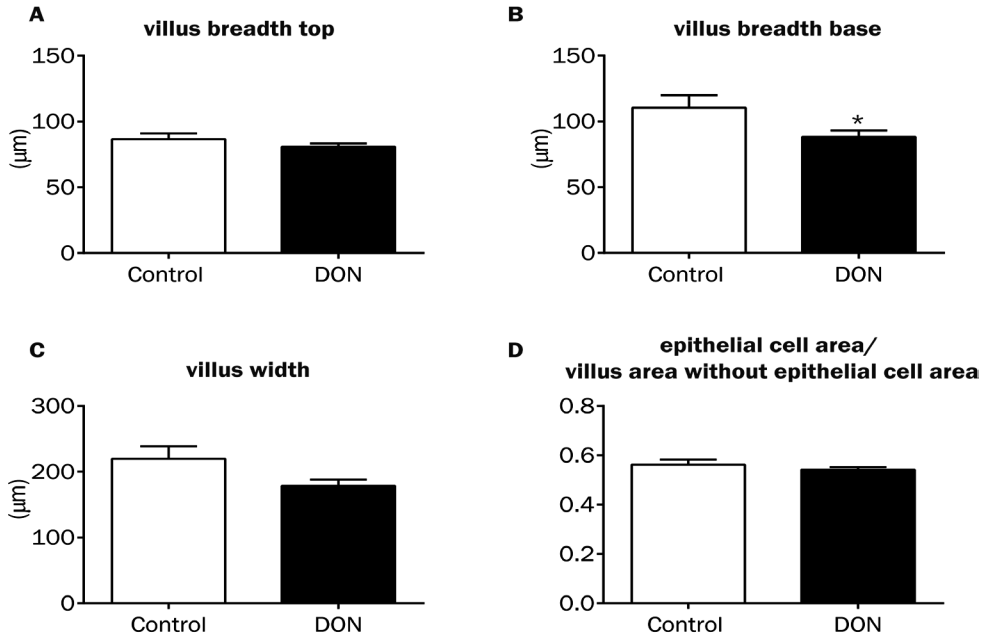
Supplementary Table 1. Feed composition of standard piglet diet.

Nutrient ratio	Value	Nutrient ratio	Value
Calcium (g)/Phosphorus (g)	1.38	Digestible methionine (g)/ Digestible lysine (g)	0.37
Calcium (g)/Digestible phosphorus flour (g)	1.87	Digestible threonine (g)/ Digestible lysine (g)	0.62
n-6 poly unsaturated fatty acid (g)/ n-3 poly unsaturated fatty acid	8.28	Digestible tryptophan (g)/ Digestible lysine (g)	0.19
Digestible methionine+cystine(g)/ Digestible lysine (g)	0.59		



Supplementary Figure 1. Histomorphological measurements in the piglet duodenum

*P ≤ 0.05; significantly different from the control group.



Supplementary Figure 1. Histomorphological measurements in the piglet jejunum

*P ≤ 0.05; significantly different from the control group.

Chapter 5

Mitigating adverse effects of deoxynivalenol in weaned piglets by dietary supplementation with non-digestible oligosaccharides: a histomorphometric analysis

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Abstract

The mycotoxin deoxynivalenol (DON) is one of the most common cereal-based food and feed contaminant worldwide. DON exposure is of particular concern in pigs related to their high sensitivity and the high percentage of wheat in pig diets. Currently, the limited efficiency of mycotoxin-mitigating agents against DON still awaits alternative solutions to reduce DON exposure in pigs. There is scarce information regarding dietary interventions which can promote gut health and strengthen the intestinal barrier to prevent the adverse effects of DON. Hence, the main objective of the current study was to investigate whether a mixture of non-digestible galacto-oligosaccharides (GOS) is able to attenuate the DON-induced histomorphological alterations in the piglet small intestine.

Piglets (10 piglets/group) received a control diet or DON diet (0.9 mg/kg feed) with or without supplementation of 0.8% GOS for 10 days and the effects on the intestinal histomorphology were investigated. Supplementation of the DON-containing diet with 0.8% GOS fully prevented the DON-induced decrease in villus area and epithelial cell area in the duodenum. In jejunum, the increase in crypt depth induced by DON could be completely prevented as well by addition of GOS to the diet.

Considering the promising effects of GOS against the DON-induced histomorphological alterations in the piglet small intestine, further investigations into additional beneficial effects of GOS in DON-exposed piglets are warranted.

Introduction

Cereal grains and their by-products are very important energy and protein sources in farm animal and human food supplies. Cereal crops are susceptible to fungal invasion and these fungi are responsible for the production of various secondary metabolites, including mycotoxins [1,2]. The trichothecene mycotoxin deoxynivalenol (DON) produced by several *Fusarium* spp. is one of the major mycotoxin contaminating cereal grains. DON contamination can cause serious economic losses worldwide, either by yield losses (*Fusarium* head blight) and reduced crop values, or by losses in animal productivity [3,4].

The adverse effects of DON on animal and human health have been investigated extensively [5,6]. DON exposure is of particular concerns in pigs due to their high sensitivity to DON and the high percentage of wheat in pig diets [5,7,8]. In pigs, typical adverse effects, due to prolonged dietary exposure to DON, are decreased weight gain, anorexia, and altered nutritional efficiency [5].

In agriculture, the preference of feed and food industries is to prevent DON contamination in the field and prior to harvesting, and therefore different control strategies, including genetic resistance, use of biological and chemical agents, and improvement of agricultural practices focus on avoiding the growth of *Fusarium* spp. and inhibiting DON synthesis [9–11]. However, under certain environmental conditions in daily practice, DON contamination of grains remains unavoidable and food and feed contamination cannot be entirely prevented [12–14]. Another strategy for reduction of animal exposure to DON and other mycotoxins is the use of the so-called mycotoxin-mitigating agents with the intention to suppress the absorption and promote the excretion (sequestering agents) or degrade and transform mycotoxins into less toxic metabolites (biotransforming agents) [15]. However, currently the limited efficiency of commercially available products against DON and some other mycotoxins still awaits alternative solutions.

There is limited information regarding dietary interventions which can promote gut health and strengthen the intestinal barrier to prevent the adverse effects of DON, although the intestine is the first barrier encountered by dietary DON exposure [16]. Evidence from our group pointed out that a mixture of non-digestible galacto-oligosaccharides (GOS), prevents the DON-induced intestinal (epithelial) barrier disruption *in vitro* and in an *in vivo* mouse model [17]. GOS are already widely used in infant formula with the aim to promote gut health as they act as a prebiotic in shaping the gut microbiota, as well as showing direct effects on the maintenance of intestinal integrity [18–21]. Moreover, we recently showed in a neonatal piglet model that a GOS-supplemented milk replacer promotes the balance of the developing intestinal microbiota, improves the intestinal architecture and seems to stimulate the intestinal defense mechanism in pigs as well [22].

Therefore, the main objective of this study was to investigate whether these GOS are able to attenuate the adverse effects of DON in piglets.

To this end, piglets were fed a DON-contaminated diet with the recommended maximal concentration of 0.9 mg/kg (in line with European Commission Recommendation 2006/576/EC) with or without supplementation of GOS (0.8%) for 10 days and the effects on the intestinal histomorphology and architecture were investigated.

Materials and Methods

Animals

Forty 4-weeks old (the weaning age: 3 weeks old) Dutch Landrace piglets from a commercial pig farm were housed under conventional conditions at the experimental farm of the Test and Training Center for Agriculture (Proef-en Vormingscentrum voor de Landbouw; PVL, Bocholt, Belgium). The animals were allocated to 4 groups (10 piglets per group) based on equal distribution of gender (4 male / 6 female) and acclimatized to the environment for 2 weeks prior to the experiments. The groups were housed in separate pens and had free access to feed and water. Temperature and lighting programs were followed standard recommendations. The trial was performed according to the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and the Belgian law on the protection of animals and the experimental protocol was agreed upon by the Ethical Committee on the use of experimental animals.

Experimental design

Four groups of ten 6-week-old piglets were fed a control (standard) diet, a control diet supplemented with 0.8% GOS (Vivinal® GOS syrup, FrieslandCampina Domo, Borculo, The Netherlands), a control diet supplemented with 0.9 mg DON/kg feed (Romer Laboratories, Inc., Union, MO, USA) or the DON diet supplemented with GOS for 10 days. The control diet consisted of a commercial standard diet for piglets (Cibus NV, Poperinge, Belgium) and no DON contamination was observed (analyzed by HPLC analysis, detection limit: 10 µg/kg). Piglets were sedated with an intramuscular injection of azaperone (Stresnil® Elanco Animal Health, Greenfield, IN, USA) (4 mg/kg) prior to the induction of euthanasia via an intravenous injection of 200 mg/kg pentobarbital (Euthasol®, Virbac Animal Health, Carros Cedex, France).

Histomorphometric Analysis of the Small Intestine

The small intestine was removed and segments from the duodenum and jejunum (approximately 10 cm in length) were collected, embedded in paraffin after fixation with 10% neutral buffered formalin and 5 µm sections were stained with haematoxylin and eosin (H&E) according to standard methods. Photomicrographs of these sections were obtained using the Olympus BX50 microscope (Olympus Europa GmbH, Hamburg, Germany) equipped with a Leica DFC 320 digital camera (Leica Microsystems, Wetzlar, Germany) and 10 randomly selected villi and crypts were chosen per piglet. A

computerized microscope-based image analyzer (Cell^D version 3.3; Olympus Europa GmbH, Hamburg, Germany, 2009) was used to determine different histomorphometric parameters, including villus height (measured from the tip of the villus to the villus-crypt junction), crypt depth (measured from the crypt villus junction to the base of the crypt), villus area (total surface of the villus) and epithelial cell area (villus surface area minus villus area without epithelial cells). All aforementioned parameters were manually defined for each villus [23,24].

Statistical analysis

Experimental results are expressed as mean ± SEM. Statistical analysis was performed by using GraphPad Prism 6.0 (Graphpad, La Jolla, CA, USA) and differences between experimental groups were statistically determined by using Two-way ANOVA followed by Bonferroni multiple comparison test. Results were considered statistically significant when $P < 0.05$.

Results

Histomorphometric analysis of the small intestine

DON exposure did not significantly decrease the villus height compared to the control piglets (Figure 1A), but resulted in a significant decrease in villus area and epithelial cell area in the duodenum (Figure 1C and 1D). Supplementation of the DON diet with 0.8% GOS fully prevented this DON-induced decrease in villus area and epithelial cell area (Figure 1C and 1D), whereas the DON+GOS fed animals showed longer villi in the duodenum compared to the DON-fed animals.

In the jejunum, DON in the diet did significantly reduce villus height, as well as the villus area and epithelial cell area compared to the controls (Figure 2A, 2C and 2D). These DON-induced alterations in jejunum were not significantly affected by GOS supplementation to the DON diet (Figure 2A, 2C and 2D). However, the increase in crypt depth in jejunum induced by DON could be completely prevented by addition of GOS to the diet (Figure 2B).

Representative photomicrographs of villi in duodenum and jejunum from piglets fed a control diet, DON diet, GOS diet and DON+GOS diet are depicted in Figure 1 and 2.

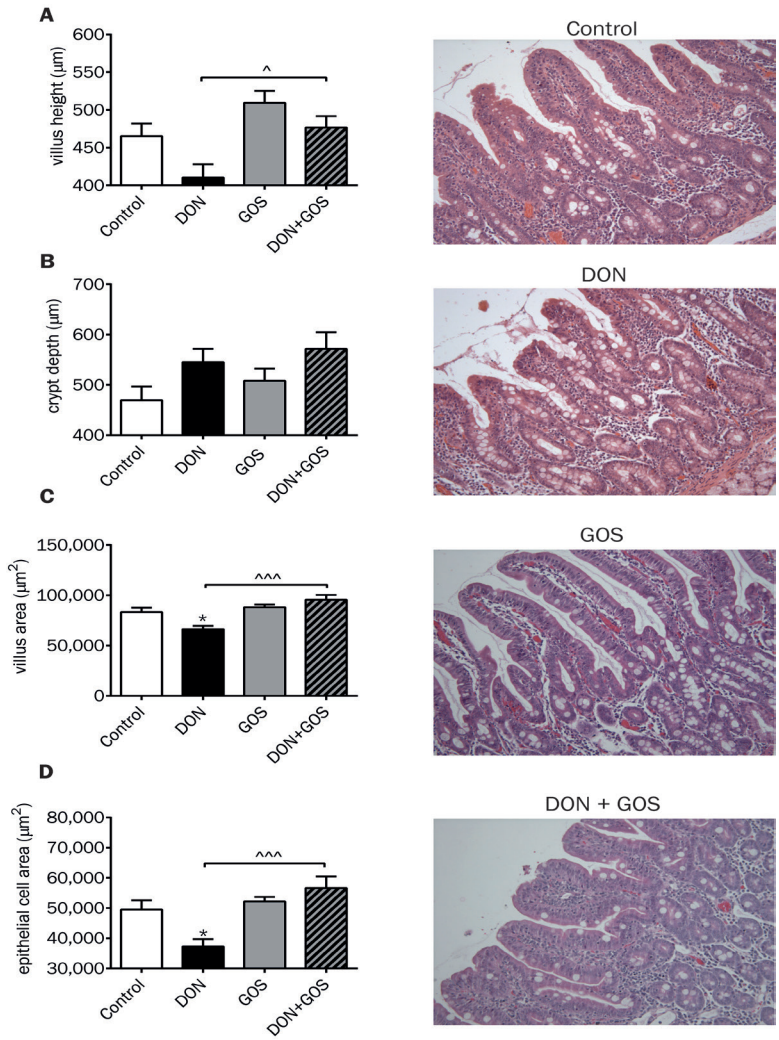

Figure 1. GOS prevent the DON-induced histomorphological alterations in the duodenum of the piglet intestine. Piglets received a control or DON diet (0.9 mg/kg feed) with or without 0.8% GOS supplementation for 10 days. Histomorphometric analysis of the villus height (A), crypt depth (B), villus, area (C) and epithelial cell area (D) in duodenum as described in material and methods; representative photomicrographs of villi in duodenum from piglets fed the different diets. Magnification 200x. Results are expressed as means ± SEM; n = 9-10 animals per group. *P ≤ 0.05; significantly different from control group, ^P < 0.05, ^^^P < 0.001; significantly different from DON group.

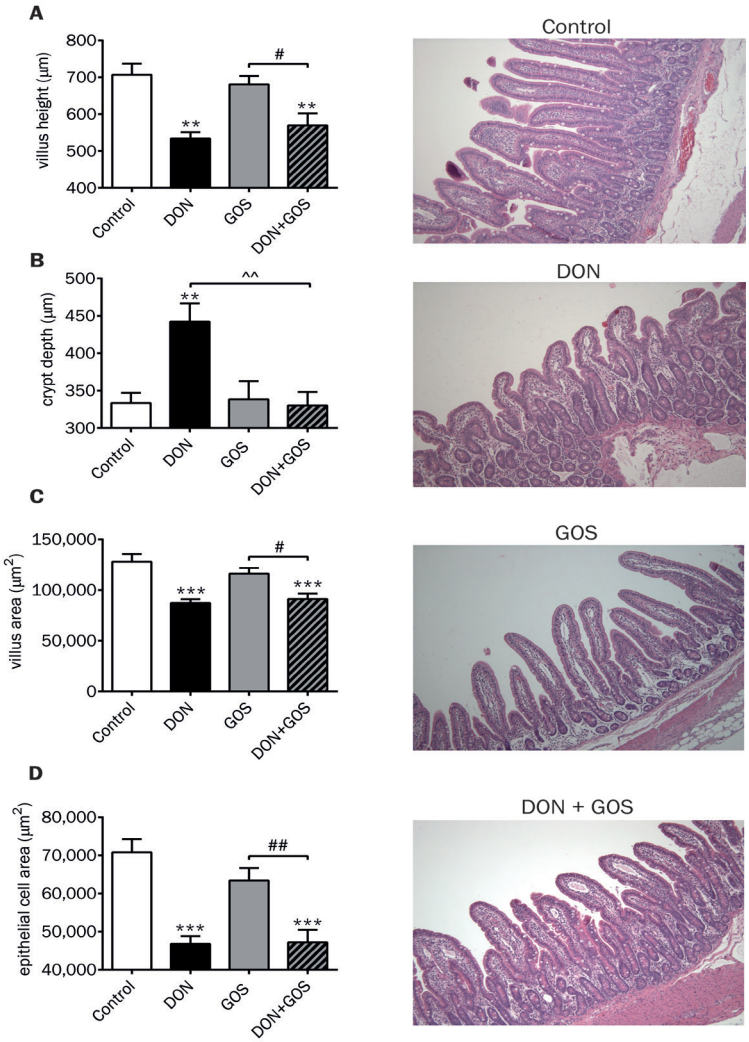

Figure 2. GOS partly prevent the DON-induced histomorphological alterations in the jejunum of the piglet intestine. Piglets received a control or DON diet (0.9 mg/kg feed) with or without 0.8% GOS supplementation for 10 days. Histomorphometric analysis of villus height (A), crypt depth (B), villus area (C) and epithelial cell area (D) in jejunum as described in material and methods; representative photomicrographs of villi in jejunum from piglets fed the different diets. Magnification 100x. Results are expressed as means ± SEM; n = 9-10 animals per group. **P < 0.01, ***P < 0.001; significantly different from control group, ^^P < 0.01; significantly different from DON group, ##P < 0.01; significantly different from GOS group.

Discussion

The aim of the current study was to investigate whether a dietary intervention with GOS, which promotes gut health and strengthening the intestinal barrier, can prevent the adverse effects of DON in piglets. The piglets received a diet with very low DON levels (0.9 mg/kg feed) for 10 days, which is the statutory recommended maximum DON concentration in feed in the European Union (EU). Recently, our group showed that even these low DON levels can induce histomorphological changes in the intestine of piglets, since the villi in duodenum and jejunum of piglets given the DON diet were proportionally smaller compared to the villi from control animals [23].

In the current study, 0.8% GOS prevented the DON-induced decrease in villus area and epithelial cell area in the duodenum of piglets. These beneficial effects of GOS are in line with previous studies from our group using various *in vitro* and *in vivo* models. More specifically, after acute DON exposure (oral gavage) in mice, the mice fed a diet with 1% GOS showed larger villi in the proximal small intestine compared to DON-exposed mice fed a control diet, most probably by preventing the villus contraction after acute mucosal injury by DON [17,25]. Recently, we reported that already after 3 days GOS, an increased villus height in the duodenum of neonatal piglets was observed, whereas in neonatal piglets fed a GOS diet for 26 days, an improved villus architecture was detected in both duodenum and jejunum [22]. Here, no significant effects on intestinal morphology were observed in the 6-week-old piglets fed a GOS supplemented diet for 10 days compared to control piglets, which could suggest that early life GOS intervention is more effective. In the current study, the increase in crypt depth in jejunum induced by DON could be completely prevented by addition of GOS to the diet. An increase in crypt depth is indicative for an increased rate of enterocyte turn-over, and their subsequent migration to the villi tips. Impairment of this process results in more immature cells with reduced disaccharidase enzyme activity [26] and previous investigations confirmed a positive correlation between villus height and disaccharidase activity in the intestines of weaning piglets [27,28]. In the previous study we observed also a significant increase in maltase activity, one of the main intestinal disaccharidases, in the caecal mucosa in piglets fed a GOS diet for 26 days compared to control animals [22]. From these findings it can be concluded that GOS stimulate intestinal health and probably also nutrient utilization.

Positive effects of other oligosaccharides on the intestinal histomorphology are previously described, since Barnes *et al.* [29] indicated that short-chain fructo-oligosaccharides treatment increased ileal villus length and stimulated intestinal adaptation in a neonatal intestinal failure piglet model. Dietary supplementation with chito-oligosaccharide and cello-oligosaccharides resulted in an increase in villus height and villus:crypt ratio in the small intestine of weaning pigs [30,31].

Regarding the mechanism of GOS to diminish the adverse effects of DON in the intestine, various hypotheses have been offered. Initially, and in consideration of some similarities with yeast cell wall-derived oligosaccharides and milk-derived oligosaccharides, a

direct interaction between GOS and DON was assumed, but such a physico-chemical interaction between DON and GOS was excluded in the study of Akbari *et al.* [17].
Considering the well-known prebiotic effects of GOS to enhance the populations of Bifidobacteria and Lactobacilli [22,32], it can be speculated that this increased bacterial population either sequesters, as described for Lactobacilli, or counteracts the adverse effects of DON on the intestinal barrier [14,33].

Conclusion

Considering the promising preventive effects of GOS on the DON-induced disruption of the intestinal barrier *in vitro* and *in vivo* [17] together with the observed effects of GOS against the DON-induced histomorphological alterations in the piglet small intestine, further investigations are warranted to unravel additional beneficial effects of GOS in DON-exposed piglets.

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Chapter 6

The piglet as a model for studying dietary components in infant diets: effects of galacto-oligosaccharides on intestinal functions

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This chapter is published in British Journal of Nutrition 2016; 115(4): 605-618

Abstract

Prebiotic oligosaccharides, including galacto-oligosaccharides (GOS), are used in infant formula to mimic human milk oligosaccharides, which are known to have an important role in the development of the intestinal microbiota and the immune system in neonates. The maturation of the intestines in piglets closely resembles that of human neonates and infants. Hence, a neonatal piglet model was used to study the multi-facetted effect of dietary GOS in early life. Naturally farrowed piglets were separated from the mother sow 24-48 h post-partum and received a milk replacer with or without addition of GOS for 3 or 26 days, whereafter several indicators of intestinal colonization and maturation were measured. Dietary GOS was readily fermented in the colon, leading to a decreased pH, an increase in butyric acid in caecum digesta, and an increase in Lactobacilli and Bifidobacteria numbers at day 26. Histomorphological changes were observed in the intestines of piglets fed a GOS diet for 3 or 26 days. In turn, differences in the intestinal disaccharidase activity were observed between control and GOS-fed piglets. The mRNA expression of various tight junction proteins was up-regulated in the intestines of piglet fed a GOS diet and was not accompanied by an increase in protein expression. GOS also increased defensin porcine β-defensin-2 (pBD-2) in the colon, and secretory IgA (sIgA) levels in saliva. In conclusion, by applying a neonatal piglet model, it could be demonstrated that a GOS-supplemented milk replacer promotes the balance of the developing intestinal microbiota, improves the intestinal architecture and seems to stimulate the intestinal defence mechanism.

Introduction

The gastrointestinal tract (GIT) has a key role in nutrient supply, and it has an impact on functions of the entire organism, as the highest number of immune cells and the highest diversity in microbiota are present in the gut [1]. The immediate postnatal period is the most critical phase in the establishment of the intestinal microbial population, and accumulating evidence suggests that this early colonization of the gut also determines the reactivity of the immune system in later phases of life [2]. For example, Sjögren *et al.* [3] showed that a more diverse microbiome early in life seems to reduce the prevalence of allergies. During the initial maturation period, the intestinal tract undergoes profound growth, as well as morphological and functional differentiation [4]. Previous data have shown that breast-fed infants benefit from the special composition of colostral and early breast milk, as they have a lower risk of developing intestinal disorders and a lower incidence and severity of diarrhoea, inflammatory reactions and atopic diseases when compared with formula-fed infants [5]. The high concentration (12-15 g/l) and structural diversity of human milk oligosaccharides (HMO) are unique to humans, and HMO represent the first prebiotics in life. Hence, prebiotic oligosaccharides, including galacto-oligosaccharides (GOS) derived from bovine milk, are added to infant formulas, alone or together with other prebiotics [6,7]. Previous data have suggested that GOS have beneficial effects on the composition of the microbiota and the priming of the infant's immune system [8,9]. In adults, GOS are also effective in alleviating symptoms of irritable bowel syndrome [10]. To allow a closer understanding of the different mechanisms involved in these beneficial effects of GOS and related substances, various animal models have been applied [11–14]. It appeared that in contrast to rodents, the postnatal gut development and nutritional requirements of piglets more closely resemble the human infant in many aspects [15–17]. The purpose of this study was to apply such a piglet model using normally delivered piglets and supplementing their standard formula diet with a fully characterized GOS mixture, to identify physiological and morphological changes in the intestine of GOS-treated piglets. To this end, at different time points in the perinatal period the effects of GOS on the fermentation, microbiota composition, brush-border enzyme activity, histomorphology of the intestinal tract, the intestinal integrity and the intestinal defence mechanism were investigated.

Materials and methods

Animals

All *in vivo* experimental protocols were approved by the Ethics Committee for Animal Experiments (Reference number: DEC 2011.III.11.117) and were performed in compliance with governmental and international guidelines on animal experimentation. The experiment was carried out with Landrace × Yorkshire piglets obtained from the faculty-owned farm. The piglets were naturally farrowed and stayed with the sow for 24-48 h post-partum to obtain maternal antibodies with the colostrum. Thereafter, forty piglets were selected from four litters and allocated to four experimental groups (10 piglets per group) with an equal distribution of littermates, weight and sex in each group. The selected piglets were transferred into a separate room with four individual pens, equipped with floor heating and additional infra-red (IR) lamps and nesting material. A 12 h light - 12 h dark cycle was used.

Diets

After weaning, all piglets received a commercial milk replacer diet (Milkiwean babymilk Yoghurt, Trouw Nutrition, Putten, The Netherlands) (Supplementary Table 1). All piglets were offered the milk diets in large plates and started unassisted drinking within 6 h after weaning. Initially, the calculated daily ration (according to the product information) was presented in six feedings per day (approximately 600 ml/piglet/day) and increased gradually to 1600 ml/piglet/day offered at four feedings per day. Two groups received the milk replacer alone, and the other two groups received the same milk replacer supplemented with 0.8% short chain GOS, which is comparable to the amount of oligosaccharides added to infant formula [18]. GOS was obtained from FrieslandCampina Domo (Vivinal® GOS syrup, 75% dry matter, Borculo, The Netherlands), which contain oligosaccharides with a degree of polymerisation (DP) of 2 - 8 with approximately 59% (w/w) GOS, 21% (w/w) lactose, 19% (w/w) glucose and 1% (w/w) galactose on dry matter. Drinking water was provided *ad libitum.*

Experimental design

From each group (controls and GOS-fed piglets) one subgroup of ten animals was killed at the age of approximately 4 days (3 days on the GOS-supplemented diet), whereas the other two subgroups (control and GOS-fed piglets) of ten animals were sacrificed at the age of approximately 27 days (26 days on the GOS-supplemented diet). Weight of the piglets was registered at weaning (day 1) and twice a week during the entire experiment. At the end of the experimental periods, the piglets were individually anesthetized with an intramuscular injection of 10 mg/kg ketamine (Narketan®, Vetoquinol, Lure Cedex, France) and 4 mg/kg azaperon (Stresnil®, Elanco Animal Health, Greenfield, IN, USA), followed by induction of euthanasia with an intra-cardiac injection of 200 mg/kg pentobarbital (Euthasol®, Virbac Animal Health, Carros Cedex, France). Blood was

collected via cardiac puncture immediately after death.

Sampling procedures and analyses

Measurement of pH in the digesta

After euthanasia, the GIT was removed and the contents of the stomach, duodenum, jejunum, ileum, caecum and colon were collected by gently squeezing the digesta from the different parts. Immediately after sampling, the pH was recorded using pH-indicator strips (Merck, Darmstadt, Germany) and the digesta samples were stored at -80˚C.

Measurement of caecal short chain fatty acids (SCFA) and lactate

Caecum digesta was weighed and homogenized in phosphate-buffered saline (PBS) (100 mg digesta/ml) using a Precellys 24 tissue homogeniser (Bertin Technologies, France) five times for 10 s at 6000 rpm with a minimum of 5 minutes cooling period on ice in between. Samples were then centrifuged for 15 minutes at 14000 rpm and the supernatant was collected and stored at -80˚C.

The caecal SCFA levels of acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid and valeric acid were quantitatively determined as described previously [19]. The SCFA were captured using a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector. SCFA concentrations were determined using 2-ethylbutyric acid (Sigma Chemical Company, St Louis, MO, USA) as an internal standard.

For lactate measurement, homogenised caecum digesta was thawed on ice and centrifuged for 5 minutes at 14000 rpm, and 100 µl of the supernatant was heated for 10 minutes at 100˚C to inactivate all enzymes. Lactate was determined enzymatically using an L-lactic acid detection kit with D- and L-lactate dehydrogenase (Boehringer Mannheim, Mannheim, Germany) [19].

Characterisation of dietary oligosaccharides

Oligosaccharides were analysed in caecal digesta and faecal samples, of control- and GOS-fed piglets, after salt and monomers removal from the samples by solid phase extraction. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was used to identify and quantify oligosaccharides, as described previously [20].

Microbiota analysis

Faecal samples were collected in sterile tubes at day 0, 12 and 26 of the intervention study, and stored directly at -80˚C. As the animals were housed as a group to guarantee the social contact between the piglets, it was not possible to link all faecal samples to the corresponding piglet within a group. DNA extraction was performed as described previously [21]. Briefly, 1 ml of a 10% (w/v) faecal slurry in anaerobic PBS (0.1 mol/l, pH 7) was prepared, homogenised for 2 minutes and centrifuged for 5 minutes at 13400 g.

Bacterial DNA was isolated from the frozen pellet using a Qiagen (West Sussex, UK) stool kit according to the manufacturer's instructions with an additional lysozyme (Sigma, 30 mg/ml Tris-EDTA)/metanolysin (Sigma, 1000 U/ml Tris-EDTA) step to aid breakdown of the cell walls. Quantitative PCR was conducted from an adapted method of Ritalahti *et al.* [22]. Briefly, 5 µl of DNA samples/standards were applied and 20 µl of mastermix solution (Applied Biosystems, Foster City, CA, USA) was added including relevant primer sets and probes with 6-carboxyfluorescein (6-FAM) as a reporter fluorophore on the 5' end, with dihydrocyclopyrroloindole tripeptide minor groove binder quencher on the 3' end [21]. For total bacteria, SYBR Green mix (Applied Biosystems) was used. Samples were analysed using the AB 7700 sequence detector (Applied Biosystems) in conjunction with Sequence Detector System software (Applied Biosystems). Total number of bacteria, Bifidobacteria, Lactobacilli, Clostridia, *Bacteroides* spp. and *Escherichia coli* (Supplementary Table 2) were determined using qPCR as described in [21,23,24]. Results are expressed as $\log_{10}$ colony-forming unit (CFU)/g faeces.

Measurement of intestinal disaccharidase activities

After killing the animals on day 26, the entire intestine was removed and washed in saline after collecting the contents of the different segments (duodenum, jejunum, ileum, caecum and colon). The mucosa of each segment was scraped off with a microscope slide and frozen in liquid nitrogen. After thawing, the mucosal scrapings were weighed, solubilized in cold PBS (200 mg/ml), homogenised with TissueLyser II (Qiagen) (homogeniser) and centrifuged for 10 minutes (3000 g) to collect the supernatants. Protein concentrations of each supernatant were determined using a Pierce® BCA Protein Assay Kit (ThermoScientic, Rochford, IL, USA). The digestive enzymes lactase, sucrase and maltase were determined by the method of Dahlqvist [25]. Briefly, this procedure consists of measuring glucose levels of homogenised mucosal scrapings incubated with the specific substrate. The substrates lactose, sucrose and maltose were dissolved to prepare 56 mM solution in 0.1 M sodium maleate buffer (pH 6.0). Glucose was used as a standard and the glucose content released during the reaction is determined by the glucose oxidase method, which allows the calculation of specific enzyme activity expressed as units (U) of disaccharidase per mg protein. One unit is defined as the activity of a disaccharidase needed to hydrolyze 1 µmol of disaccharide per minute.

Histomorphometric analysis of pig intestines

The small intestine parts (duodenum, jejunum and ileum) were fixed in 10% neutral buffered formalin, embedded in paraffin and 5 µm sections were cut and stained with haematoxylin/eosin (H&E) according to standard methods. Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera. The morphometric analysis of the sections was performed on 10 randomly selected, well-oriented villi and crypts per animal. A computerized microscope-based image analyser (Cell^D, Olympus, Europa GmbH, Germany) was used to determine histomorphometric

parameters: villus height (measured from the tip of the villus to the villus-crypt junction), villus breadth top, villus breadth base, villus width (measured at the bottom of villi), crypt depth (measured from the crypt-villus junction to the base of the crypt), villus surface area (total surface of the villus) and epithelial cell area (villus surface area minus villus area without epithelial layer). These regions of interest were manually defined for each villi separately.

qRT-PCR analysis of intestinal tissue samples

For mRNA studies, the pig intestine was flushed with cold PBS and separated into different segments. These segments were defined as follows: duodenum, jejunum, ileum, caecum and colon. These whole intestinal wall samples (approximately 2-3 cm) were snap frozen in liquid nitrogen and stored at -80°C for RNA isolation. 50 mg of each sample was suspended into 350 µl RNA lysis buffer with β-mercaptoethanol and homogenized using a TissueLyser (Qiagen, Hilden, Germany) for 1 minute/25 Hz. Total RNA was isolated using spin columns according to the manufacturer's instructions (Promega, Madison, WI, USA). Subsequently, 1 µg of extracted total RNA was reversely transcribed with the iScriptTM cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The iQSYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used for the qRT-PCR according to manufacturer's instructions. qRT-PCR was performed using the MyIQ single-colour real-time PCR detection system (Bio-Rad, CA, USA) and MyIQ System Software Version 1.0.410 (Bio-Rad Laboratories Inc., CA, USA). The PCR cycle parameters were: general denaturation at 95°C for 3 minutes, 1 cycle, followed by 40 cycles of 95°C for 20 s, annealing temperature (AT) for 30 s, and elongation at 72°C for 30 s. Primer sequences for claudin-1, -2, -3, -4, occludin, zona occludens protein-1 (ZO-1), ZO-2, porcine β-defensin-1 (pBD-1), pBD-2, pBD-3, porcine epididymis protein 2 splicing variant C (pEP2C) and protegrins 1-5 (PG1-5) with corresponding annealing temperatures are listed in Supplementary Table 3 and were derived from the NCBI GenBank and were manufactured commercially (Eurogentec, Seraing, Belgium). Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as reference gene, since HPRT1 is a good reference gene for transcripts in different pig tissues [26]. No significant effect of GOS treatment on the Ct values of HPRT compared to the control animals was observed (data not shown).

Western blot analysis

Approximately 50 mg of intestinal samples (duodenum and colon) were lysed using 500 µl RIPA lysis buffer (Thermo scientific, Rockford, IL, USA) with protease inhibitors (Roche Applied Science, Penzberg, Germany), and the total protein concentration was measured by the BCA protein assay kit (Thermoscientific, Rockford, IL, USA). Standardized protein amounts of boiled samples were isolated by electrophoresis (CriterionTM Gel, 4%–20% Tris-HCL, Bio-Rad Laboratories, Hercules, CA, USA) and electro-transferred onto polyvinylidene difluoride membranes (Bio-Rad, Veenendaal,

The Netherlands). 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 occludin (1:1000, Abcam, Cambridge, UK), ZO-1 (1:1000, Invitrogen, Carlsbad, CA, USA) or claudin-1 (1:1000, Invitrogen, Carlsbad, CA, USA). After washing in PBST, the membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (1:5000, Dako, Glostrup, Denmark) for 2 h 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 Laboratories, Hercules, CA, USA). Subsequently, the membranes were re-probed with a β-Actin antibody (1:4000, Cell Signaling, Danvers, MA, USA) to assess the equality of loading. Signal intensities were quantified using the ImageJ 1.47 software (National institutes of Health, Bethesda, MD, USA), and the protein expression was normalized with β-Actin and expressed as the mean fold change in relation to the control group.

Measurement of ZO-1 in serum samples

Serum was derived from blood (~10 ml), harvested by centrifugation (15 minutes at 1500 × g) in BD vacutainer tubes (BD, Plymouth, UK) and stored at -20°C. The ZO-1 levels in serum samples were measured by ELISA according to manufacturer's instructions (Elabscience, Wuhan, China).

Measurement of sIgA in saliva

Saliva was collected using Salivette tubes (Sarstedt, Nümbrecht, Germany) containing a synthetic swab. The sampled pigs were allowed to chew the swab prior to feeding time at day 0, 12, 15, 19, 22 and 26, until thoroughly moist. The swabs were then placed in test tubes and centrifuged at 3000 g for 10 minutes. The saliva samples were removed and stored at -20°C until analysis. The sIgA levels in the saliva samples were measured by ELISA according to manufacturer's instructions (MyBioSource.com, San Diego, CA, USA).

Statistical analysis

Experimental results are expressed as mean ± SEM. Analyses were performed by using GraphPad Prism (version 6.01) (GraphPad, La Jolla, CA, USA). Differences between groups were statistically determined by using an unpaired two-tailed student's t-test or a Two-way analysis of variance (ANOVA) with Bonferroni post-hoc test. Microbiota data are displayed on a logarithmic scale. Results were considered statistically significant when $P < 0.05$. Changes in relative mRNA expression between groups are described when an expression ratio of two-fold or higher is observed [27].

Results

No effect of GOS on animal health and body weight

The piglets remained healthy during the experimental period and no diarrhoea was observed in response to the dietary treatment. No significant increase in body weight was observed after 3 days GOS diet (control: 10.4 ± 2.0% increase *versus* GOS: 11.1 ± 1.3% increase) or after 26 days GOS diet (control: 341.8 ± 16.5% increase *versus* GOS: 345.4 ± 11.9% increase).

GOS modulate the intestinal microbiota

The total microbial population in the faeces was quantified at day 0, 12 and 26 of the experiment (26-day experiment) using qPCR and converting the results into $\log_{10}$ CFU/g faeces (Figure 1). Total bacterial counts remained almost constant and no significant differences were observed between controls and GOS-supplemented diets (Figure 1A). Next to total faecal counts, several bacterial groups including, *Bacteroides*, Lactobacilli, Bifidobacteria, *E. coli* and Clostridia were quantified in the faeces. *Bacteroides* counts were increased on day 0 prior to the onset of GOS feeding and seem to reflect inter-individual variability (Figure 1B). Lactobacilli were significantly increased in the GOS-diet group at day 26 (Figure 1C), and a similar increase on day 26 was observed for *Bifidobacterium* spp. (Figure 1D). In addition, the increase in Bifidobacteria was not significant different when using a primer set for Bifidobacteria analysis in multiple species (data not shown). *E. coli* and Clostridia counts decreased over time (Figure 1E and 1F), and Clostridia could not be detected at day 12 and 26. Although, the number of *E.coli* bacteria tended to decrease in the GOS treated animals at day 12, no significant differences between treatment groups could be detected.

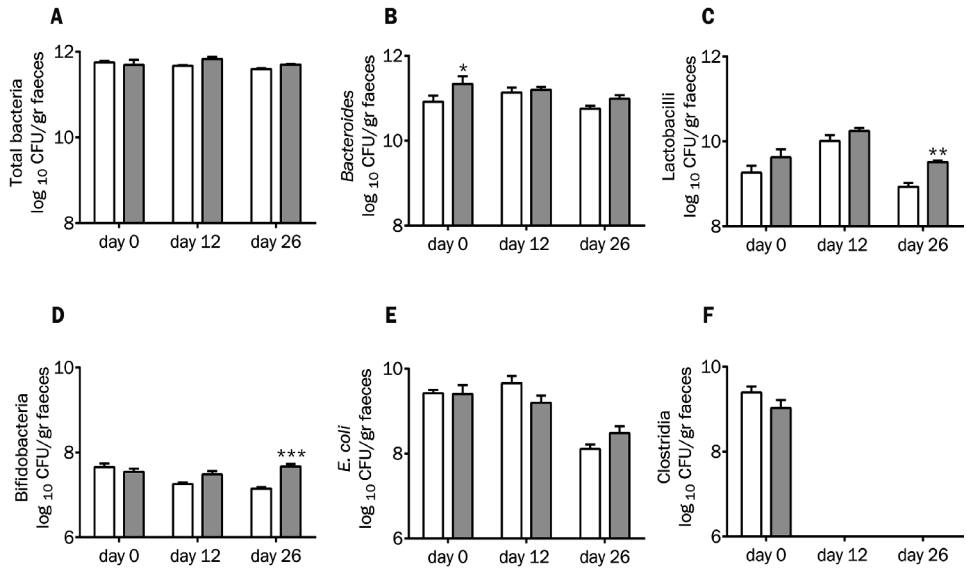

Figure 1. Galacto-oligosaccharides (GOS) modulate the intestinal microbiota. Faeces of control (white bars) and GOS-fed piglets (grey bars) were collected at day 0, 12 and 26 and the composition of the intestinal microbiota was determined by qPCR as described in material and methods. n= 3-9 animals per group. Bacterial numbers are expressed as $\log_{10}$ colony forming units (CFU)/g faeces ± SEM. (two-way ANOVA with Bonferroni post-hoc test; *P < 0.05, **P < 0.01, ***P < 0.001; significantly different from the corresponding control group).

The pH in caecal digesta is significantly decreased after 26 days GOS in the diet

A decrease in pH value of the caecum digesta was observed in piglets fed the GOS diet for 3 days compared to the control (pH 6.1 ± 0.27 *versus* pH 6.7 ± 0.26, respectively) (Figure 2A), however this effect was not significant and less pronounced than the effect for the GOS diet after 26 days. Significantly lower pH values were observed in the caecum digesta of piglets fed the GOS diet for 26 days (pH 5.9 ± 0.11) as compared with piglets fed the control diet (pH 7.1 ± 0.32) (Figure 2B). The pH in the content of the stomach, duodenum, jejunum, ileum and colon was not significantly affected by the experimental diet (Figure 2A and 2B).

Dietary GOS lead to an increase in butyric acid in caecum digesta

Although the short chain fatty acid concentrations were not significantly affected by GOS after the 3-day exposure period, butyric acid tended to increase in caecum digesta (control: 0.90 ± 0.13 mmol/l *versus* GOS: 1.22 ± 0.25 mmol/l) (Figure 2C). At 26 days, a significantly higher molar concentration of butyric acid was measured (control: 0.95 ± 0.08 mmol/l *versus* GOS: 1.28 ± 0.10 mmol/l) (Figure 2D), whereas no significant

differences were observed in the concentrations of acetic acid, propionic acid, and valeric acid in the caecum digesta after GOS diet for 26 days. Iso-butyric acid and iso-valeric acid were not detectable in the caecum digesta and D- and L-lactate levels were below the detection limit (0.2 mmol/l) after 26 days control or GOS diet and no significant differences could be observed after 3 days GOS (D-lactate; control: 0.579 ± SEM 0.136 mmol/l *versus* GOS: 0.523 ± SEM 0.135 mmol/l and L-lactate; control: 1.025 ± SEM 0.267 mmol/l *versus* GOS: 0.838 ± SEM 0.214 mmol/l).

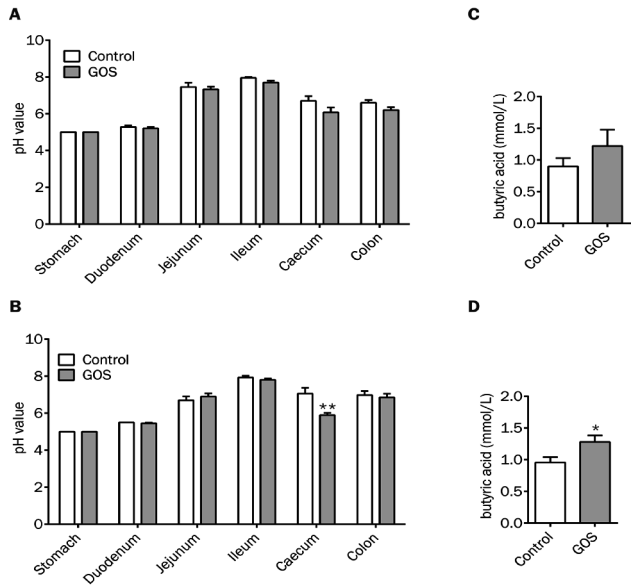

Figure 2. Dietary GOS lead to a pH decrease and an increase in butyric acid in caecum digesta. On day 3 (2A and 2C) and 26 (2B and 2D) the control and GOS-fed piglets were sacrificed and the content of the stomach, duodenum, jejunum, ileum, caecum and colon was collected for pH analysis (2A and 2C) and butyric acid measurement (2B and 2D) as described in materials and methods. n = 10 animals per group. Values are expressed in pH value (pH analysis) or mmol/L (butyric acid measurement) as mean ± SEM. (unpaired two-tailed student's t-test; *P < 0.05, **P < 0.01; significantly different from the control group).

No intact GOS structures are present in faecal samples from piglets fed a GOS diet

The presence of oligosaccharides derived from the GOS diets was investigated in the piglet caecal and faecal samples. A low abundance of oligosaccharides was detected in faecal samples of piglets fed a GOS diet for 3 days (Figure 3). These oligosaccharides predominantly represent DP2 and DP3 and were not recognised as intact GOS structures. After 26 days GOS diet, in caecal digesta samples a low abundance of oligosaccharides was detected as well, while in faecal samples hardly any oligosaccharides were present (Figure 3). As expected, in the faecal samples of piglets fed the control diet for 3 days or 26 days no quantifiable oligosaccharides were present (data not shown).

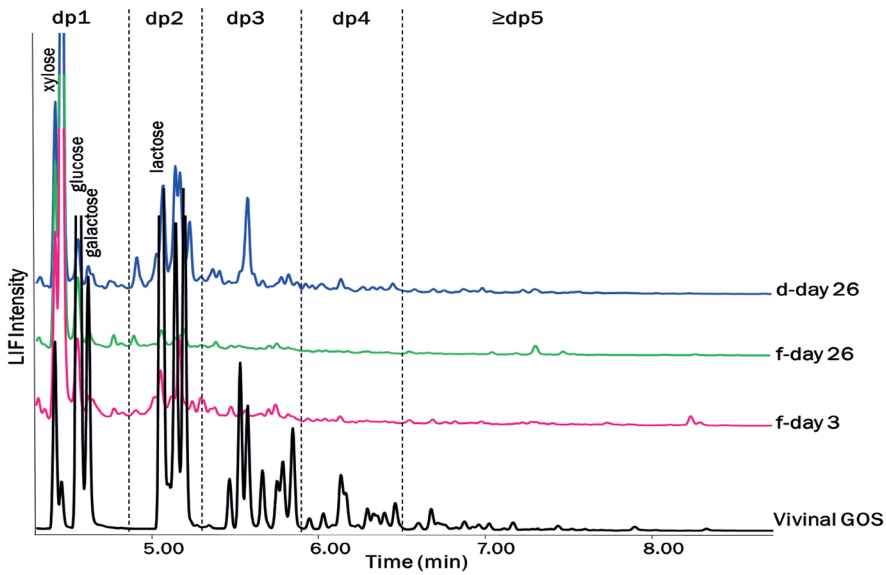

Figure 3. No intact GOS structures are present in faecal samples from piglets fed a GOS diet. Capillary electrophoresis (CE) with laser induced fluorescence (LIF) detection profiles of vivinal GOS, and of oligosaccharides as detected in faecal samples of piglets fed a GOS diet for 3 and 26 days (f-day 3, and f-day 26), and in caecal digesta samples of piglet fed a GOS diet for 26 days (d-day 26).

GOS induce histomorphological changes in the intestine of piglets

Histomorphometric analysis of the small intestine (duodenum, jejunum and ileum) of piglets was performed after 3 days and 26 days GOS diet. Table 1 and Table 2 show the results on the small intestinal histology (villus height, villus breadth top, villus breadth base, crypt depth, villus area, villus area without epithelial cell area, epithelial cell area) in relation to the type of diet and small intestinal segment. Already after 3 days histomorphological differences were observed in the duodenum of the piglets fed a GOS diet as the villi were increased in height and the mucosa area was enlarged (Table 1).

Table 1. Intestinal morphology of piglets fed a control or GOS diet for 3 days†

	Duodenum				**Jejunum**				**Ileum**			
	Control		GOS		Control		GOS		Control		GOS	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Villus height	427	31	**637***	72	642	53	613	56	417	33	473	61
Villus breadth top	47	2	48	2	53	2	51	2	52	2	51	2
Villus breadth base	69	4	70	4	65	3	66	4	72	4	66	3
Villus width	143	7	147	9	135	5	136	8	151	9	132	5
Crypt depth	327	20	278	14	224	12	212	7	237	12	216	11
Villus area	63554	5952	**93885***	10085	99988	10485	90255	8723	67446	4772	70849	9827
Villus area without epithelial cell area	27264	3097	**47255****	5771	40570	4893	38019	4018	31192	2801	34626	5472
Epithelial cell area	36291	3235	46630	4486	59418	6042	52236	5565	36254	2441	36223	4423
Villus crypt ratio	1	0.1	**2.3****	0.1	3	0.2	3	0.3	2	0.1	2	0.2

GOS, galacto-oligosacharides.

* The difference between the means of control and GOS fed groups is statistically significant (*P < 0.05, ** P < 0.01).

† Analysed by using an unpaired two-tailed student's t-test.

The value for villus height, villus breadth top and base, villus width and crypt depth are presented as (μm)

The value for villus area, villus area without epithelial cell area and epithelial cell area are presented as (μm^2)

The villi in the duodenum of piglets given the GOS diet for 26 days were proportionally thicker, since the villus width and the villus breadth base were significantly increased in the GOS-fed piglets (Table 2). Also the jejunum of piglets fed a GOS diet for 26 days showed thicker and larger villi as an increase in villus height, villus breadth top and villus breadth base was measured (Table 2). No differences in ileum were observed after 3 days or 26 days GOS diet.

Table 2. Intestinal morphology of piglets fed a control or GOS diet for 26 days†

	Duodenum				**Jejunum**				**Ileum**			
	Control		GOS		Control		GOS		Control		GOS	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Villus height	719	63	780	50	837	65	**1014 ***	59	501	20	583	42
Villus breadth top	78	9	76	3	72	3	**81 ****	2	75	5	82	3
Villus breadth base	116	5	**131 ***	3	107	4	**121***	3	106	6	119	4
Villus width	243	11	**270 ***	6	225	11	247	6	220	11	243	8
Crypt depth	433	27	487	29	345	18	348	17	278	14	313	22
Villus area	165617	18026	189861	14697	180235	20632	224946	16371	102588	6154	123002	11829
Villus area without epithelial cell area	66249	10284	77410	7923	75643	9405	98518	8340	44778	2858	55320	6686
Epithelial cell area	99378	8423	112451	7124	104592	11787	126428	9036	57811	3725	68119	6044
Villus crypt ratio	2	0.2	2	0.2	2	0.2	3	0.2	2	0.1	2	0.1

GOS, galacto-oligosacharides.

* The difference between the means of control and GOS fed groups is statistically significant (*$P < 0.05$, ** $P < 0.01$).

† Analysed by using an unpaired two-tailed student's t-test.

The value for villus height, villus breadth top and base, villus width and crypt depth are presented as (µm)

The value for villus area, villus area without epithelial cell area and epithelial cell area are presented as (μm^2)

GOS modulate the activity of disaccharidases

The disaccharidase activity increased with time in all segments of the intestines (Table 3 and 4). GOS in the diet for 3 days did not significantly affect the low lactase and maltase activity levels in the intestinal mucosa, except for a slight increase in maltase activity in the colon of the GOS-fed group compared to the control piglets (Table 3).

Table 3. Intestinal disaccharidase activities at 3 days†

	Lactase (U/mg)		Maltase (U/mg)	
	Mean	SEM	Mean	SEM
Duodenum				
Control	0.753	0.059	0.142	0.017
GOS	0.665	0.089	0.119	0.015
Jejunum				
Control	1.080	0.058	0.254	0.054
GOS	1.123	0.059	0.294	0.067
Ileum				
Control	0.884	0.121	0.232	0.027
GOS	0.728	0.123	0.310	0.041
Caecum				
Control	0.246	0.084	0.150	0.037
GOS	0.156	0.043	0.135	0.020
Colon				
Control	0.227	0.089	0.155	0.010
GOS	0.368	0.119	**0.188***	0.009

GOS, galacto-oligosacharides.

* The difference between the means of control and GOS fed groups is statistically significant (*P < 0.05)

† Analysed by using an unpaired two-tailed student's t-test.

The jejunal mucosa of piglets at day 26 was associated with the highest lactase, sucrase and maltase activities, whereas the caecum and colon contained the lowest brush border enzyme activity (Table 4). Duodenal and jejunal disaccharidase activities were similar between control piglets and piglets fed a GOS diet for 26 days, but ileal lactase, sucrase and maltase activity were lower in piglets fed a GOS diet compared to the controls (Table 4). Lactase and sucrase activity were not detectable in caecal and colonic mucosa after 26 days. The maltase activity in caecal mucosa was significantly increased in piglets fed a GOS diet for 26 days compared to control animals (Table 4).

Table 4. Intestinal disaccharidase activities at 26 days†

	Lactase (U/mg)		**Sucrase (U/mg)**		**Maltase (U/mg)**	
	Mean	SEM	Mean	SEM	Mean	SEM
Duodenum						
Control	1.122	0.261	0.119	0.062	1.304	0.241
GOS	0.929	0.132	0.062	0.027	0.890	0.089
Jejunum						
Control	5.027	0.832	6.143	0.839	9.855	0.762
GOS	4.022	0.869	4.512	0.896	8.012	0.645
Ileum						
Control	0.754	0.169	3.450	0.634	6.980	0.629
GOS	**0.319***	0.039	**1.864***	0.408	**4.723***	0.668
Caecum						
Control	ND	-	ND	-	0.174	0.021
GOS	ND	-	ND	-	**0.506***	0.123
Colon						
Control	ND	-	ND	-	0.249	0.053
GOS	ND	-	ND	-	0.236	0.045

GOS, galacto-oligosacharides.

* The difference between the means of control and GOS fed groups is statistically significant (*P < 0.05)

† Analysed by using an unpaired two-tailed student's t-test.

ND: Not determined

The mRNA and protein expression of different tight junction proteins are increased by dietary GOS

The potential effect of GOS on the mRNA expression and protein levels of tight junction (TJ) proteins were measured by qRT-PCR as well as by Western blot analysis. Interestingly, already after 3 days GOS, different TJ proteins were up-regulated in different parts of the intestine (Figure 4A, 4C, 4E, 4G and 4I). In the duodenum, jejunum, ileum and caecum of piglets fed with a GOS diet enhanced mRNA levels of claudin1 (CLDN1) were detected, and ZO-1 and ZO-2 mRNA expression levels were increased in the caecum and colon after 3 days of GOS diet. No significant differences were detected at TJ protein level after 3 days of control or GOS diet (Figure 5 and supplementary Figure 1).

GOS supplemented to the diet for 26 days enhanced the mRNA levels for OCLN, ZO-1 and ZO-2 in the duodenum, whereas in the jejunum, caecum and colon the mRNA levels of only CLDN1 were up-regulated (Figure 4B, 4D, 4F, 4H and 4J). Furthermore, the OCLN protein expression was significantly increased in the colon of GOS-fed piglets compared with control piglets at 26 days (Figure 5E), but the CLDN1 and ZO-1 protein expression did not significantly differ between control and GOS-fed piglets (Figure 5A, 5D, 5C, 5F and supplementary Figure 1). GOS did not remarkably affect the mRNA expression of CLDN2, -3 and -4 in different parts of the intestine. Besides diet effects, an increase in OCLN and ZO-1 protein expression was observed in the duodenum of GOS-fed animals at 26 days compared to the GOS-fed piglets at 3 days, (Figure 5B and 5C). In addition, no differences were detected in ZO-1 serum levels between the control and GOS-fed piglets (Supplementary Figure 2). However, the ZO-1 serum levels were significantly decreased in the piglets from the 26-day study compared with the piglets used in the 3-day study.

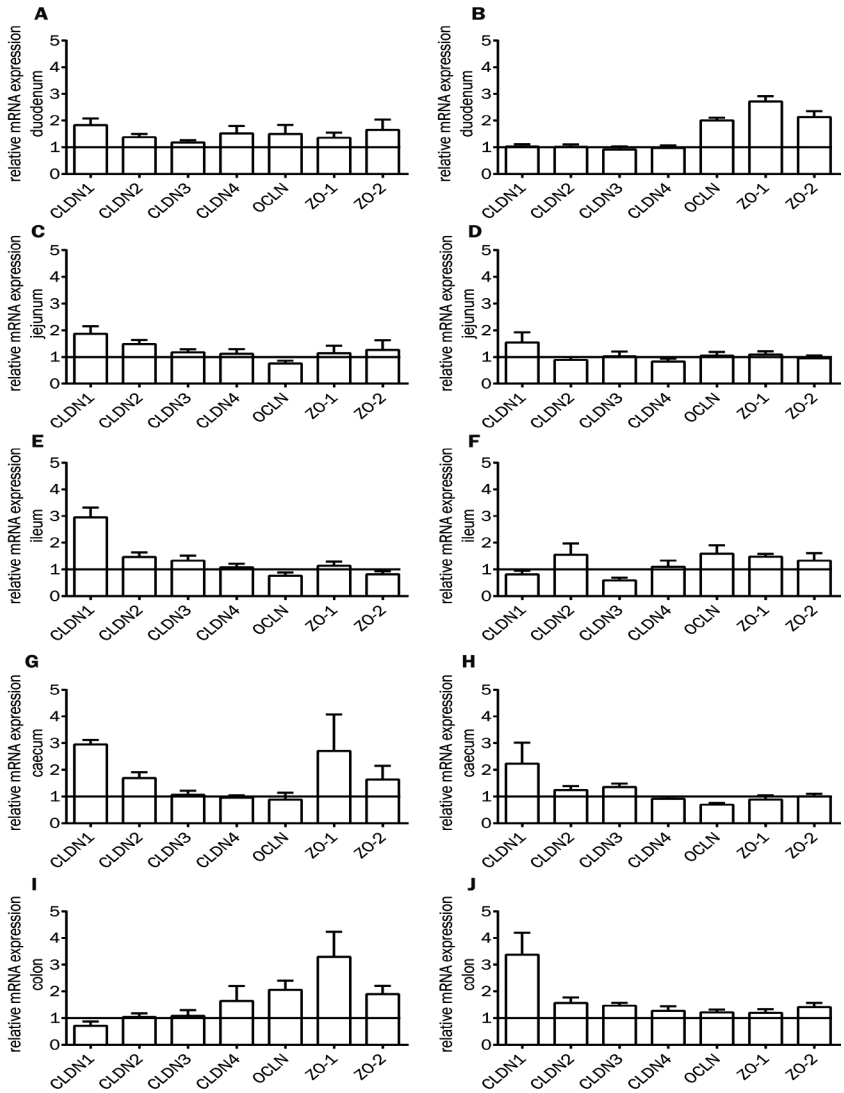

Figure 4. The mRNA expression levels of different tight junction proteins are up-regulated by dietary GOS. Piglets received a control or GOS diet for 3 or 26 days and samples from different parts of the intestine (duodenum (4A and 4B), jejunum (4C and 4D), ileum (4E and 4F), caecum (4G and 4H) and colon (4I and 4J)) were collected and mRNA levels of tight junction proteins (CLDN1, -2, -3, -4, OCLN, ZO-1 and ZO-2) were measured by qRT-PCR. n = 10 animals per group. Results are expressed as relative mRNA expression (fold of control, normalized to HPRT1) as mean ± SEM. The horizontal line represents the control group.

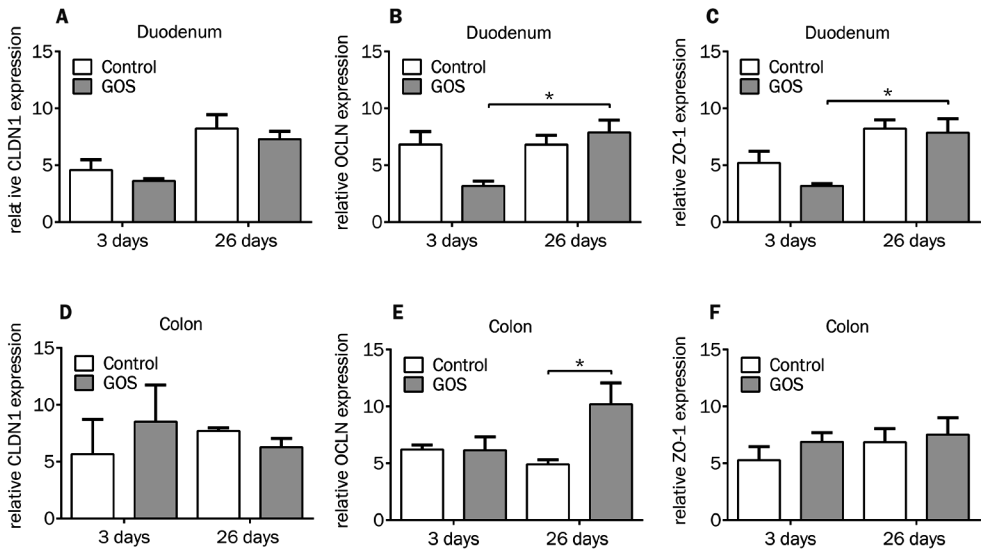

Figure 5. The protein levels of different tight junction proteins are increased by dietary GOS. Piglets received a control or GOS diet for 3 or 26 days and samples from different parts of the intestine (duodenum (5A, 5B and 5C) and colon (5D, 5E and 5F)) were collected and tight junction proteins levels (CLDN1, OCLN and ZO-1) were measured by Western Blot analysis. n = 4 animals per group. Results are expressed as relative protein expression (optical density normalized with β-actin) as mean ± SEM. (two-way ANOVA with Bonferroni post-hoc test; *P < 0.05).

Defensin pBD-2 mRNA expression levels are increased by dietary GOS

mRNA levels of defensins, antimicrobial peptides secreted by the colonic epithelium, were measured in the colon of piglets fed the GOS diet for 3 and 26 days compared with piglets fed the control diet (Figure 6). The mRNA expression of pBD-2 was increased in the colon of piglets fed a GOS diet for 3 days (Figure 6A) compared with the control piglets, but this effect was absent in the piglets fed a GOS diet for 26 days (Figure 6B). No differences were detected in the mRNA expression of the other defensins, pBD-1, pBD-3, pEP2C and PG1-5 between GOS-treated and control animals.

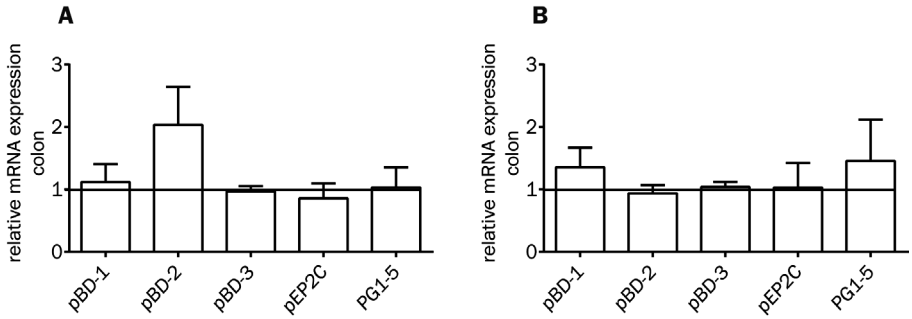

Figure 6. Defensin pBD-2 mRNA expression levels are increased by dietary GOS. Piglets received a control or GOS diet for 3 or 26 days, and samples from the colon were collected and mRNA levels of defensins (pBD-1, pBD-2, pBD-3, porcine epididymis protein 2 splicing variant C (pEP2C) and protegrins 1-5 (PG1-5)) were measured by qRT-PCR. n = 10 animals per group. Results are expressed as relative mRNA expression (fold of control, normalized to HPRT1) as mean ± SEM. The horizontal line represents the control group.

sIgA levels in saliva are increased by dietary GOS

The effects of GOS on the mucosal immune system were investigated by measuring sIgA in saliva. At day 19, 22 and 26, the addition of GOS in the diet significantly increased the salivary IgA levels (39.0%, 20.1% and 25.2% increase, respectively) compared to the piglets fed with a control diet (Figure 7). At the earlier time points (0, 12 and 15 days) the salivary concentrations of IgA remained unaffected by GOS (Figure 7).

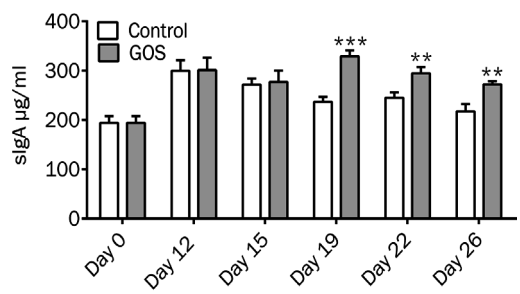

Figure 7. Secretory Immunoglobulin A (sIgA) levels in saliva are increased by dietary GOS. Saliva of control and GOS-fed piglets was collected at different time points day 0, 12, 15, 19, 22 and 26 and the sIgA levels were measured by ELISA. n = 8-10 animals per group. Values are expressed in µg/ml as mean ± SEM. (Two-way ANOVA with Bonferroni post-hoc test; **P < 0.01, ***P < 0.001; significantly different from the control group).

Discussion

It is known that piglets share many characteristics with human infants in the perinatal development of their intestinal tract [17]. Hence, one of the main objectives of this study was to establish a model with neonatal piglets suitable for studying the effects of early life dietary interventions on intestinal development and function. In the current study, two different diets were compared: a commercial milk formula for piglets with or without GOS. The selection of GOS as model compounds was based on the fact that these oligosaccharides are currently the most widely used oligosaccharides in infant formulas and are known to have gut-modulating properties [28].

A major difference between piglets and human newborns is the level of protection by maternal antibodies at birth. Human newborns are protected in the first period of life by placentally transferred maternal antibodies [29], whereas new-born piglets are fully dependent on acquiring maternal antibodies from passive immunization via colostrum because of the poor passage rate of antibodies through the placenta [30]. Therefore, in this *in vivo* model, the piglets stayed with the sow to obtain colostrum during the first 24-48 h. This implies, however, that the primary colonisation of the intestinal flora followed a natural pattern for piglets, and differences in composition of the microflora because of dietary oligosaccharides might be less pronounced when compared with infants.

Related to the early suckling period, the composition of the intestinal microbiota varied already between animals after 24-48 h of life, before the onset of the formula feeding (day 0). This was expected as the piglets originated from different sows and therefore the micro-environment during the immediate postnatal period was slightly different. Data by Thompson *et al.* [31] showed a high level of individuality in 1- and 2-week-old piglets, suggesting a considerable randomness to the process of acquiring microbes. This rapid colonisation of the intestinal tract is also reflected by the finding that bacterial quantities in the faeces at day 0 were already comparable with the numbers at day 12 and 26. Previous investigations already indicated that within 12 h after birth bacterial densities could stabilise at 10^9 - 10^{10} bacteria/g colonic content of the pig and within 48 h 90% of the microflora can be constituted [32]. It is also known that the bacterial colonisation in the intestine of breastfed infants is stabilised within the first few days of life [33]. Differences between the two diets, composed of a standard pig formula and the same formula supplemented with GOS, showed particularly differences in the prevalence of Lactobacilli and Bifidobacteria, which is in line with clinical trials including human infants [9].

The high bacteria density present at the first stages of life, in addition to increased bifidobacterial numbers in the intestine of the GOS-fed piglets, may explain the low abundance of intact GOS structures in caecal samples, as well as their absence in the faeces of GOS-fed piglets. GOS might have a high stability in the small intestines, as the presence of GOS in serum and urine demonstrates their selective adsorption in the porcine intestinal tract [34].

Results from clinical studies indicated that GOS are fermented by the bacterial flora in the human colon [35]. De Leoz *et al.* [36] suggested that in premature human infants at least a portion of the GOS passes through the intestinal tract undigested by the intestinal microbiota. On the other hand, an *in vitro* study conducted in a fermentation screening platform showed that adult human inocula were able to ferment a broader variety of dietary fibres than pig inocula [37]. *In vitro*, GOS was fermented by adult human microbiota, thereby producing acetate, propionate, butyrate, succinate and lactate [38]. Clinical studies with humans showed elevated levels of acetate, butyrate and lactate in faecal samples after oligosaccharides diets, which was associated with acidic faecal pH values [39–41]. Walton *et al.* [21] concluded that the elevated Bifidobacteria numbers account for the increased butyrate production observed after GOS supplementation. Higher butyric acid production was also observed in the faeces of formula (including GOS and FOS)-fed infants compared to breast fed infants [40]. In the current study, caecal acetic acid, propionic acid, valeric acid and lactate concentrations did not differ significantly between the control and GOS-fed piglets, but the concentration of butyric acid was significantly increased. As the overall levels of butyric acid remained low, butyric acid might not be the only factor that contributes to lower the caecal pH values in the GOS-fed piglets. Butyric acid has been shown to prevent the colonisation of various pathogens such as *E. coli* [42] and dietary butyrates are described to inhibit inflammation and to improve the gut barrier function [43].

Besides effects on the large intestine, GOS can also exert positive effects on the gut morphology in the small intestine. The pattern of porcine small intestinal development is comparable to that reported for the human small intestine, in contrast to for example rodents, and the villus development in pigs occur at similar relative times in gestation when compared with humans [17,44]. The increased villus surface area and villus:crypt ratio in the duodenum induced by a GOS diet already within 3 days and the increased villus surface area in duodenum and jejunum after 26 days GOS can be related to improved utilisation of nutrients [45]. In humans and pigs, very little is known about the effects of oligosaccharides on gut morphology early in life, and the exact mechanism for the changes in villus surface is still unclear. Previous investigations on ileal histomorphology demonstrated that villus height was increased in neonatal piglets receiving formula + GOS via enteral feeding compared to parenterally fed piglets, but no differences were found when compared with enterally fed piglets given formula alone [46]. FOS treatment increased ileal villus length compared with control in a neonatal intestinal failure piglet model [11], whereas dietary supplementation of chito- and cello-oligosaccharide increased the villus height in the small intestine of weaning pigs [47,48]. The presence of GOS might prevent villus shortening occuring after early weaning in pigs [49,50], rather than increasing the villus surface area. Tsukahara *et al.* [51] found a positive correlation between villus height and disaccharidase activity in the small intestine of piglets. These digestive enzymes are located in the brush-border membrane and facilitate nutrient utilisation. Both in newborn humans and piglets lactase activity is

elevated during birth [50], but pigs differ from humans by being born with low activities of disaccharidases that are necessary for hydrolysis of non-lactose carbohydrates [52]. Indeed, we observed low maltase activity levels, whereas the sucrase activity was not even measurable at day 3. In humans, the absolute disaccharidase activity levels tend to be higher in human jejunum and ileum compared to duodenum [53], which was also observed in our piglet study at 26 days. Although the maltase levels were low in the caecum at day 26, the increased maltase levels in the GOS group might convey an advantage for the post-weaning adaptation to diets for growing piglets, as these diets are mainly composed of starch. As lactose was mainly present in the commercial milk replacer, the reduction in ileal brush-border enzyme activity observed in piglets fed a GOS diet for 26 days may result in an increased passage and availability of lactose to the large intestine, resulting in an increased risk for osmotic diarrhea [54]. Diarrhoea was not observed in our study, in which a dosage of 0.8% GOS was used. These undesirable effects are likely to be dose-dependent, as diarrhoea and flatulence were observed in adults only after consumption of more than 15 g prebiotics per day [55].

In addition to nutrient digestion and absorption, the gastrointestinal epithelium also serves as a physical barrier against potentially harmful stressors that enter the intestinal tract, including bacteria, toxins and viruses, as well as undesirable substances in nutrients. Therefore, intestinal epithelial cells are connected by TJs, which seal the apical intercellular space [56]. To our knowledge, no information about TJ expression patterns in human neonates is available, as clinical studies mainly focus on increased intestinal permeability and abnormal expression of TJ proteins related to the pathogenesis of various gastrointestinal diseases, such as inflammatory bowel disease [57]. The intestinal barrier function and the TJ network in the pig intestine is mainly investigated around weaning time, when the intestinal barrier is challenged by rapid feed changes and infections [58]. We studied here the intestinal TJ patterns in healthy piglets and showed a slight up-regulation of the mRNA expression of different TJ proteins in the intestines of GOS-fed piglets, which was not accompanied by an increase in protein expression, except an increased OCLN protein expression in the colon by dietary GOS given for 26 days. In neonatal mammals, the gastrointestinal permeability is known to be enhanced during the first few days [59,60], which is in line with our data, as OCLN and ZO-1 protein levels were lower in the duodenum from GOS-fed piglets at day 3 compared with day 26. The higher serum levels of ZO-1 after 3 days in piglets fed a control or GOS diet may be considered as a marker of the incomplete barrier functions. This hypothesis is enforced by lower ZO-1 serum levels at the 26-days measurements, where a higher degree of maturity is achieved. Previously, we could demonstrate in an *in vitro* model with Caco-2 cells, that GOS also directly protect the intestinal barrier integrity by stimulating the TJ assembly [61], and in addition another *in vitro* study suggest that fermentation products of prebiotics, such as butyrate, are able to prevent disruption of the intestinal epithelial barrier [62]. Cani *et al.* [63] proposed that a selective gut microbiota modulation by prebiotics consequently improves gut barrier function, including improved TJs, by a

glucagon-like peptide-2 (GLP-2) dependent mechanism.
Next to the mechanical barrier, endogenous antimicrobial peptides, such as defensins, protect the animal from invading pathogens. Defensins are already expressed during pregnancy in the fetal gut, which indicates their important role in the innate immune competence during early life [64]. Lactobacilli improve gut barrier function *in vitro* via induction of defensins [65]. The increase in pBD-2 as measured in the colon of piglets fed a GOS diet for 3 days might therefore have a protective effects as well, contributing to the suppression of microbial infections or bacterial outgrowth, as suggested in previous experiments showing a concentration-dependent inhibition of the growth of bacteria following the application of synthetic pBD-2 in pigs [66]. Another component of the innate immune system in the intestine is secretory IgA, which binds harmful antigens at mucosal surfaces and neutralises toxins and virulence factors [67]. Salivary IgA is mainly produced by the mucosa associated lymphoid tissue (MALT) and its concentration reflects MALT activity [68]. Different studies indicate a strong link between the colonisation of the intestines, the development of the mucosal immune system and the production of endogenous sIgA [69,70]. In infants, especially Bifidobacteria seems to be important for the synthesis of endogenous sIgA [71], probably by affecting the development of the IgA-producing plasmablasts [72]. Monitoring of salivary IgA showed increased levels in GOS-fed piglets on days 19, 22 and 26, which can be considered as an improvement of the mucosal immune system. Promoting the abundance of specific intestinal Bifidobacteria by dietary GOS as observed in our study might contribute to this elevated sIgA concentration in the saliva. However, a direct effect on the microbiome in the oral cavity could not be entirely excluded. Higher faecal sIgA concentrations were also observed in infants fed with formula containing the prebiotic mixture GOS/FOS [73]. As formula-fed infants lack the transfer of protective maternal sIgA and antibacterial peptides from breast milk [74], they would potentially benefit from dietary ingredients that support the production of endogenous sIgA and β-defensins.
In conclusion, although there are some physiological differences in the perinatal development of their intestinal tract between neonatal piglets and human newborns, the results of this study qualify the piglet as a valid model in paediatric research for the assessment of dietary intervention strategies. Assessment of a number of key parameters show that readily fermentable GOS stimulates the development of the intestinal microbiota, improves the intestinal architecture, and seems to modulate barrier integrity and parameters of the innate immune system. The current experiments were conducted with healthy animals, and no challenge models were included. Further studies should aim to demonstrate differences in the intestinal integrity and immune competence of GOS-fed animals in the presence of infectious or chemical stressors relevant in the early phase of life in animals and humans.

Acknowledgements

The authors are grateful to all CCC members for the discussions and feedback during the meetings. The authors would like to thank Gerrit Witte (Nutricia Research) for technical assistance during SCFA measurement, Helmie van Dijck and Arjen Nauta (FrieslandCampina) for microbiota analyses and discussions on results. The authors thank M.A.M. Oosterveer-van der Doelen and J.L. de Nijs-Tjon for their technical assistance. This project is jointly financed by the European Union, European Regional Development Fund and The Ministry of Economic Affairs, Agriculture and Innovation, Peaks in the Delta, the Municipality of Groningen, the Provinces of Groningen, Fryslân and Drenthe, the Dutch Carbohydrate Competence Center (CCC WP25; www.cccresearch.nl), Nutricia Research and FrieslandCampina. E. D. and S. B. were granted by the Carbohydrate Competence Center (CCC) programme as indicated in acknowledgements; J. G. is associated with Nutricia Research and L. H. U. and M. H. C. S. with FrieslandCampina, respectively, which are both industrial partners in the Dutch Carbohydrate Competence Center.

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Supplementary data

Supplementary Table 1. Feed composition

Name	Unit	End product
Dry matter	g/kg	965.00
Moisture	g/kg	35.00
Crude protein	g/kg	200.00
Crude fat	g/kg	200.00
Crude ash	g/kg	58.00
Lactose	g/kg	325.00
Crude fiber	g/kg	0.10
Lysine	g/kg	17.00
Methionine	g/kg	5.50
Methionine + cysteine	g/kg	9.70
Threonine	g/kg	10.00
Tryptophan	g/kg	2.60
Calcium	g/kg	3.50
Phosphorus	g/kg	4.80
Sodium	g/kg	3.80
Chlorine	g/kg	7.60
Potassium	g/kg	13.00
Na+K-Cl	meq/kg	284.00
Vitamin A	mg/kg	12.00
Vitamin D_3	mg/kg	0.125
Vitamin E (all-rac-alpha-tocopherylacetate)	mg/kg	300.00
Potassiumiodide, iodine	mg/kg	1.00
Sodiumselenite, selenium	mg/kg	0.30
Copper (II) sulfate, pentahydrate, Copper	mg/kg	140.00
Manganese (II) sulfate, monohydrate, Manganese	mg/kg	45.00
Zincsulfate, monohydrate, Zinc	mg/kg	84.00
Iron (II) sulfate, monohydrate, Iron	mg/kg	80.00
Butylatedhydroxyanisole (BHA)	mg/kg	3.00
Propylgallate	mg/kg	1.00
Bacillus licheniformis + Bacillus subtilis (ratio : 1/1)	CFU/kg	2000000000.0
Net energy (NE)	kcal	3465.00

Colony form unit (CFU), Milliequivalents (meq).

Supplementary Table 2. Group and species-specific 16S rRNA gene-targeted primers and probes†

Target organism	Names of primers and probes	Sequence of primers and probes (5'-3')	References
Total bacteria	prF19totbac	TCCTAGGGAGGCAGCAGT	[21]
	prR26totbac	GGACTACCAGGGTATCTAATCCTGTT	
Bifidobacteria	prF21bifido	CCTTACCTGGGCTTGACATGT	[23]
	prR21bifido	GACGTAAGGGGCATGATGATC	
	probe A	6-fam-TCGCCCCGTGTTGC-mgb	
	probe B	6-fam-TCGCCGCATGTTGC-mgb	
Lactobacilli	prF22lactobacil	AGTGGAACTCCATGTGTAGCGG	[24]
	prR26lactobacil	GGTATCTAATCCTGTTCGCTACCCAT	
	P25lactobacil	6-fam-CGTAGATATATGGAAGAACACCAGT-mgb	
Escherichia coli	prF23Ecoli	CCTTACCTGGTCTTGACATCCAC	[21]
	prR26Ecoli	AGTTTATCACTGGCAGTCTCCTTTGA	
	P20Ecoli	6-fam-TTATCCTTTGTTGCCAGCGG-mgb	
Clostridia*	prFclosB	GGCGGACGGGTGAGTAACA	[21]
	prRclosK	CGTTGCTGCATCAGGGTTT	
	PclosF	6-fam-CGAAAGGAAGATTAATACCG-mgb	
Bacteroides	prF20bacteriode	GAGGAACCTTACCCGGGHTT	[21]
	prR17bacteriode	GGACGTAAGGGCCGTGC	
	P19bacteriode	6-fam-CTTAAGTGCCATAACGAGC-mgb	

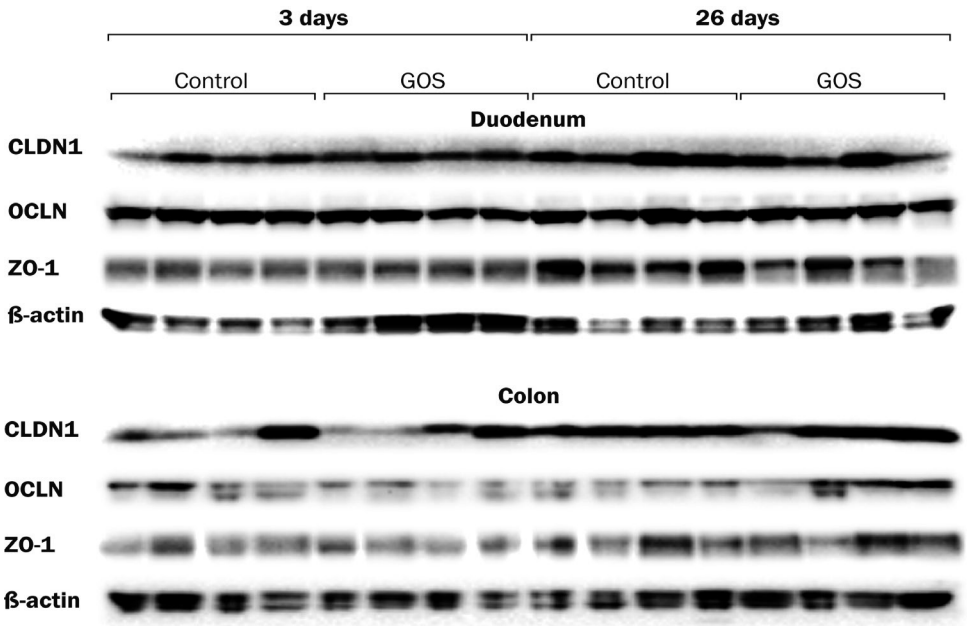
*Clostridia includes (*C. perfringens, C. paraputrificum, C. Tertium, C. Butyricum*).

† Primers used in the present study were designed by Nauta *et al.* [23,24]

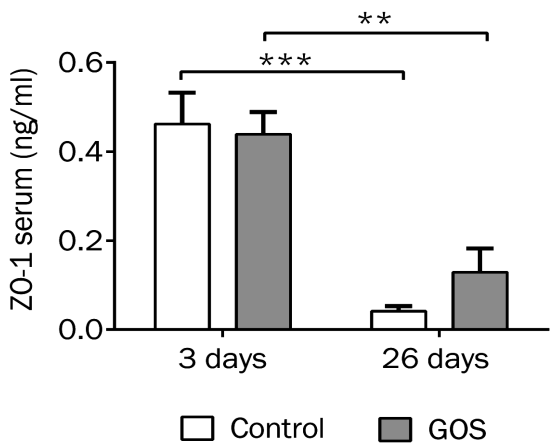
Supplementary Table 3. Primer sequences of genes used for qRT-PCR analysis

	Primer sequence (5'-3')			
Target gene	**Forward**	**Reverse**	**AT**	**References**
CLDN1	TGGCTCCGCGTCTCAGTCC	TGCGAGGGGTGCAGGTCTAA	65	NM_001244539.1
CLDN2	CTCGTTGGCCTGTATCATCACC	CAGGGGGGAGTAGAAGTCCC	63.1	NM_001161638.1
CLDN3	AACACCATCATCCGGGACTTC	CGCGGAGTAGAGGATCTTGG	61.2	NM_001160075.1
CLDN4	AGGAGAGACGCTTCAATCGG	GTCCAGACACCTGAACACCG	63.1	NM_001161637.1
HPRT	CTGAACGGCTTGCTCGAGAT	TCCAGCAGGTCAGCAAAGAA	63.1	NM_001032376.2
OCLN	ATCAACAAAGGCAACTCT	GCAGCAGCCATGTACTCT	55.8	NM_001163647.2
pBD-1	TTCCTCCTCATGGTCCTGTT	AGGTGCCGATCTGTTTCATC	58.7	NM_213838.1
pBD-2	TGTCTGCCTCCTCTCTTCC	AACAGGTCCCTTCAATCCTG	58.7	NM_214442.2
pBD-3	CCTTCTCTTTGCCTTGCTCTT	GCCACTCACAGAACAGCTACC	57	NM_214444.1
pEP2C	CCCTGGACAAGAAACAAACAA	TGACATCTGCCTTCACTTCTC	55	BK005522.1
PG1-5	GTAGGTTCTGCGTCTGTGTCG	CAAATCCTTCACCGTCTACCA	65	XM_005669497.1
ZO-1	GAGTTTGATAGTGGCGTT	GTGGGAGGATGCTGTTGT	58.7	XM_005659811.1
ZO-2	GCAGAGACAACCCCCACTTT	CGTTAACCATGACCACCCGA	55.8	NM_001206404.1

Claudin (CLDN), Hypoxanthine phosphoribosyltransferase (HPRT), occludin (OCLN), porcine β-defensin (pBD), porcine epididymis protein 2 splicing variant C (pEP2C), protegrins 1-5 (PG1-5), zona occludens protein (ZO).



Supplementary Figure 1. Western blot analysis CLDN1, OCLN, ZO-1 and β-actin. Piglets received a control or GOS diet for 3 or 26 days and duodenal and colonic tissue samples were collected. n = 4 animals per group



Supplementary Figure 2. ZO-1 serum levels. Piglets received a control or GOS diet for 3 or 26 days and serum was collected for ZO-1 measurement by ELISA. n = 8-10 animals per group. Values are expressed in ng/ml as mean ± SEM. (Two-way ANOVA with Bonferroni post-hoc test; **P < 0.01, ***P < 0.001).

Chapter 7

Direct effects of galacto-oligosaccharides on intestinal epithelial barrier integrity and epithelial cell proliferation after long-term exposure to a Caco-2 monolayer

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Abstract

The gastrointestinal barrier is immature and very susceptible to luminal antigens, pathogens and toxins in the first period of life. Therefore, non-digestible oligosaccharides, including galacto-oligosaccharides (GOS), have been developed with the aim to improve the intestinal microbiota composition early in life. The prebiotic effects of these non-digestible oligosaccharides on gut health are mainly explained through microbiota-dependent mechanisms. The aim of the current study was to investigate direct, microbiota-independent effects of GOS on the intestinal epithelial integrity and epithelial proliferation using a long-term cell culture system with pre-confluent Caco-2 cells, which resembles the situation in the fetal and neonatal intestine. Pre-confluent Caco-2 cells were exposed to different concentrations GOS (0.5%, 1% and 2%) from the apical and basolateral sides for 17 days. The epithelial integrity was increased in the GOS-treated cells as measured by an increased transepithelial electrical resistance and enhanced mRNA expression of the tight junction proteins, claudin1 and claudin3. GOS also increased the epithelial proliferation, evidenced by an upregulation of Ki67 and cyclin D1 mRNA expression and a slight increase in Ki67- and cyclin D1-positive cells.
In conclusion, GOS induced microbiota-independent effects on intestinal epithelial integrity and epithelial proliferation contributing to the development of the intestinal barrier in early phases of life.

Introduction

The prebiotic concept, defined as "selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host" [1], has received increasing attention within the last decade. Non-digestible oligosaccharides fulfill the criteria for classification as a prebiotic, including neither hydrolyzed nor absorbed in upper parts of the gastrointestinal tract, fermented by intestinal microflora and selectively stimulate beneficial, intestinal bacteria [2].

Human milk is considered to be unique in terms of its content of complex oligosaccharides [3] and these human milk oligosaccharides (HMO) are the first prebiotics that infants encounter through breastfeeding in the early days of life [4]. Because human-milk feeding cannot always be guaranteed and analogues of HMO are not available now and in the near future, alternative oligosaccharides from available sources have to provide nutritional and functional properties as close as possible to those of HMO [5]. On the basis of scientific insights from human-milk research, specific mixtures of non-digestible oligosaccharides, including galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), have been developed with the aim to improve the intestinal microbiota composition early in life [6]. This early postnatal period is the most critical phase in the establishment of the gut microbial communities and this initial colonization of the gut determines the development and reactivity of the immune system later in life [7–9].

Besides the well-known effects of these non-digestible oligosaccharides (GOS/FOS) on the composition and activity of the intestinal microbiota [6], limited studies are available investigating the microbiota-independent actions, aiming to extend the current knowledge about the direct effect of oligosaccharides on intestinal epithelial cells [10–13].

Intestinal epithelial cells are the first cell types exposed to oligosaccharides after ingestion. This single epithelial layer lining the gastrointestinal tract forms a selective barrier that can sense and respond to different stimuli to reinforce their barrier function and to participate in the regulation of appropriate mucosal immune responses [14,15]. The intestinal epithelium maintains the selective barrier function via a protein network with junctional complexes, including tight junctions (TJ), adherens junctions (AJ) and desmosomes, linking adjacent epithelial cells and sealing the intercellular space [16–18].

However, the maturation of the intestinal barrier occurs during pre- and postnatal phases [19], the gastrointestinal barrier is immature and very susceptible to luminal antigens, pathogens and toxins in the first period of life [20]. Colostrum and milk feeding have been shown to promote the maturation of the developing intestinal epithelium [21–23] and it has been demonstrated that HMO are able to improve the epithelial barrier function [24] .

Our group and others demonstrated that the non-digestible GOS have the potency to

promote the intestinal barrier function using different *in vivo* models [25–27] as well as *in vitro* Caco-2 cell systems [27,28]. Nevertheless, the direct *in vitro* effects of GOS on the intestinal epithelial layer focused on short-term exposures (24 h-48 h) and did not investigate the role of GOS during the epithelial cell maturation and differentiation phase in pre-confluent epithelial cells. Hence, the current study aimed to examine the direct effects of GOS on epithelial cell proliferation and intestinal epithelial integrity focusing on the expression of TJ junctions, AJ junctions and desmosomes using a long-term cell culture system with pre-confluent human epithelial colorectal adenocarcinoma (Caco-2) cells. Under pre-confluent conditions, these well-characterized epithelial cells differentiate in a moderate way, however upon reaching confluence, Caco-2 cells spontaneously undergo gradual villus-like enterocytic differentiation similar to that observed in the epithelium of the intact fetal and neonatal intestine [29].

Materials and Methods

The Caco-2 cell model

Caco-2 cells were obtained from the American Type Tissue Collection (Code HTB-37) (Manassas, VA, USA, passage 79-91) and were cultured as described previously [30]. The experiments were conducted with Caco-2 cells grown 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 cells were seeded at a density of 0.3 x 10^5 cells/insert in Dulbecco's Modified Eagle Medium (DMEM) (control group) or in DMEM supplemented with different concentrations GOS (0.5%, 1% and 2%) (GOS group). The Caco-2 cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C and refreshed every 2-3 days during 17 days.

GOS

The commercially available Vivinal® GOS syrup (FrieslandCampina Domo, Borculo, The Netherlands), comprising 45% GOS with 2-8 saccharide units (degree of polymerization) 16% free lactose, 14% glucose, and 25% water, was used. The GOS cytotoxicity was evaluated previously showing no cytotoxicity induced by GOS in Caco-2 cells at the mentioned concentrations [27].

TEER measurement

The transepithelial electrical resistance (TEER) was measured by a Millicell-ERS Volt-Ohmmeter (Millipore, Temecula, CA, USA) at different time points during the culturing period (17 days). Results are expressed as the percentage of the initial value [30].

RNA extraction and quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The mRNA expression of different target genes for TJ, AJ and desmosomes, including claudin (CLDN)1, CLDN3, CLDN4, zona occludens protein 1 (ZO-1) and ZO-2, occludin (OCLN), epithelial cadherin (E-cadherin), β-catenin, desmoglein 2 (DSG 2), desmoplakin 1/2 (DSP 1/2), as well as proliferation markers, cyclin D1 and Ki67 were measured by qRT-PCR. Harvesting the cells, total RNA extraction, cDNA preparation and qRT-PCR analysis were conducted according to the previously described method [30]. Forward and reverse sequences of different genes of interest are depicted in Table 1. The β-actin was used as the reference gene.

Table 1. Human primer sequences used for qRT-PCR analysis

	Primer sequence (5'-3')			
Target gene	**Forward**	**Reverse**	**AT**	**References**
DSG2	ATCAATGCAACAGATGCAGATGA	TGTCAAAGTGTAGCTGCTGTGT	65	[31]
DSP 1/2	GAAATATCTGGCAAACGAGACA	GCCAGCTGGAGCTCATAATC	65	[32]
β-catenin	AAAATGGCAGTGCGTTTAG	TTTGAAGGCAGTCTGTCGTA	64.5	[33]
E-cadherin	TGGACCGAGAGAGTTTCCCT	CCCTTGTACGTGGTGGGATT	60	[28]
Cyclin D1	GCCGAGAAGCTGTGCATCTAC	TGAGCTTGTTCACCAGGAGC	55	[34]
Ki67	CGGACTTTGGGTGCGACTT	GTCGACCCCGCTCCTTTT	58	[35]
CLDN1	AGCTGGCTGAGACACTGAAGA	GAGAGGAAGGCACTGAACCA	63	[27]
CLDN3	CTGCTCTGCTGCTCGTGTC	CGTAGTCCTTGCGGTCGTAG	63	[27]
CLDN4	GTCTGCCTGCATCTCCTCTGT	CCTCTAAACCCGTCCATCCA	62.5	[27]
OCLN	TTGGATAAAGAATTGGATGACT	ACTGCTTGCAATGATTCTTCT	57	[27]
ZO-1	GAATGATGGTTGGTATGGTGCG	TCAGAAGTGTGTCTACTGTCCG	55.8	[27]
ZO-2	GCCAAAACCCAGAACAAAGA	ACTGCTCTCTCCCACCTCCT	65	[27]
β-actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	63	[27]

AT, annealing temperature (˚C)

Immunofluorescence staining of Caco-2 monolayers

The Caco-2 cells were fixed with 10% formalin and after washing with PBS, cells were permeabilized for 5 minutes with 0.1% Triton-X-100 and blocked with 5% serum for 30 minutes. Subsequently, the samples were incubated (2 h, room temperature) with different primary antibodies directed against CLDN1 and CLDN3 (Invitrogen, Carlsbad, CA, USA), cyclin D1 (1:50, MA5-12702, Thermo Fisher Scientific, Rockford, IL, USA) and Ki67 (1:50, ab66155, Abcam, Cambridge, UK) diluted in PBS containing 1% BSA. Cells were washed with PBS and incubated with Alexa-Fluor conjugated secondary antibodies (Thermo Fisher Scientific, Rockford, IL, USA) for 1 h at room temperature. Nuclear counterstaining was conducted by incubating the samples with Hoechst 33342 (1:2000, Thermo Fisher Scientific, Rockford, IL, USA) for 3 minutes. Thereafter, the inserts were rinsed and mounted with Prolong Gold anti-fade reagent. Immunolocalization of CLDN1, CLDN3, cyclin D1 and Ki67 was visualized and images were taken using the Nikon Eclipse TE2000-U microscope equipped with a Nikon Digital Sight DS-U1 camera.

Western blot analysis

Caco-2 cell were washed twice with cold PBS and were lysed using 50 µl RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0) (Thermo Fisher Scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany). The cells were harvested and centrifuged (14.000 g for 15 minutes) and total protein concentration was determined using the BCA protein assay according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL, USA). Equal protein amounts of heat-denaturated non-reduced samples were separated

by electrophoresis (Criterion™ Gel, 4–20% Tris– HCl, Bio-Rad, Hercules, CA, USA) and electrotransferred onto polyvinylidene difluoridemembrane (Bio-Rad, Veenendaal, The Netherlands). Membranes were blocked with PBS containing 0.05% Tween-20 (PBST) and 5% milk proteins (1 h at room temperature). Subsequently, membranes were incubated with the primary antibodies directed against CLDN1, CLDN3 (1:1000, Thermo Fisher Scientific, Rockford, IL, USA) and cyclin D1 (1:1000; MA5-12702, Thermo Fisher Scientific, Rockford, IL, USA) overnight at 4˚C followed by washing the membranes in PBST. In order to assess the equality of loading, membranes were probed with the β-actin antibody (1:4000, Cell Signaling, Danvers, MA, USA). Appropriate horseradish peroxidase conjugated secondary antibodies (1:2000, Dako, Glostrup, Denmark) were applied for 2 h at room temperature. Membranes were washed in PBST and incubated with ECL Prime western blotting and digital images were obtained with the ChemiDoc MP imager (Bio-Rad Laboratories Inc.). Signal intensities were quantified using the ImageJ version 1.47 software (National institutes of Health, Bethesda, MD, USA) and the protein expression was normalized with β-actin and expressed as the mean fold change in relation to the control group.

Statistical analysis

Experimental results are expressed as mean ± SEM. Analyses were performed by using GraphPad Prism (version 6.05) (GraphPad, La Jolla, CA, USA). Differences between groups were statistically determined by using One-way ANOVA with Bonferroni post hoc test. Results were considered statistically significant when $P < 0.05$.

Results

Effects of GOS on TEER of a Caco-2 cell monolayer

Exposure of the Caco-2 monolayer to increasing concentrations of GOS (0.5%, 1% and 2%) added to the apical and basolateral side for 17 days resulted in a significant increase in TEER values compared to the control group (Figure 1). The GOS-induced TEER-increase started from day 15 of culture and only the highest concentration of GOS (2%) was effective, whereas 0.5% and 1% GOS did not significantly affect TEER values (Figure 1). In addition, after 3 days exposure of 2% GOS a slight, but significant decrease in TEER values was observed.

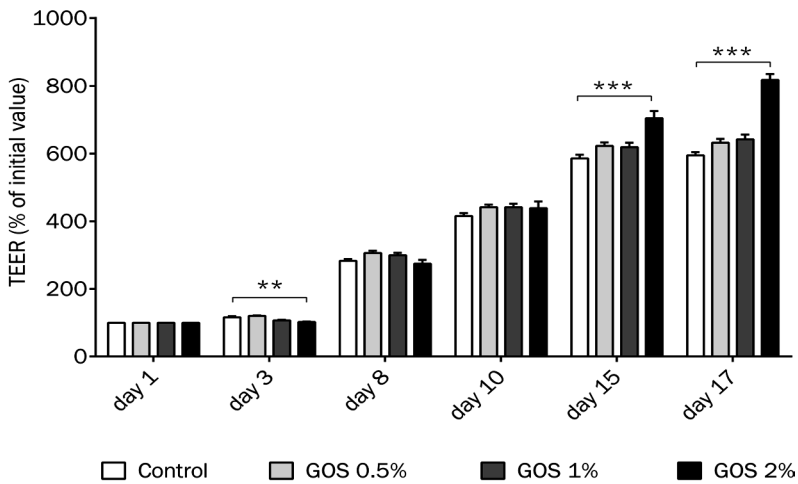

Figure 1. Effects of GOS on TEER of a Caco-2 cell monolayer. Caco-2 cells were grown on inserts with increasing concentrations GOS (0.5, 1 and 2%) on the apical and basolateral sides during 17 days. TEER was measured at day 1, 3, 8, 10, 15 and 17 of culture. Results are expressed as a percentage of initial value as mean ± SEM of three independent experiments, each performed in triplicate (**P < 0.01, ***P < 0.001; significantly different from unstimulated cells).

Effects of GOS on the mRNA expression of TJ proteins

Treatment with 1% and 2% GOS significantly up-regulated mRNA expression of CLDN1 in Caco-2 monolayers, whereas 0.5% GOS induced a significant down-regulation of CLDN1 mRNA expression compared to untreated cells (Figure 2A). Moreover, 2% GOS significantly increased the CLDN3 mRNA expression after 17 days incubation (Figure 2B). No significant changes were detected in the mRNA expression of other TJ proteins, CLDN4, OCLN, ZO-1 and ZO-2, in response to GOS incubation (Figure 2C, 2D, 2E and 2F).

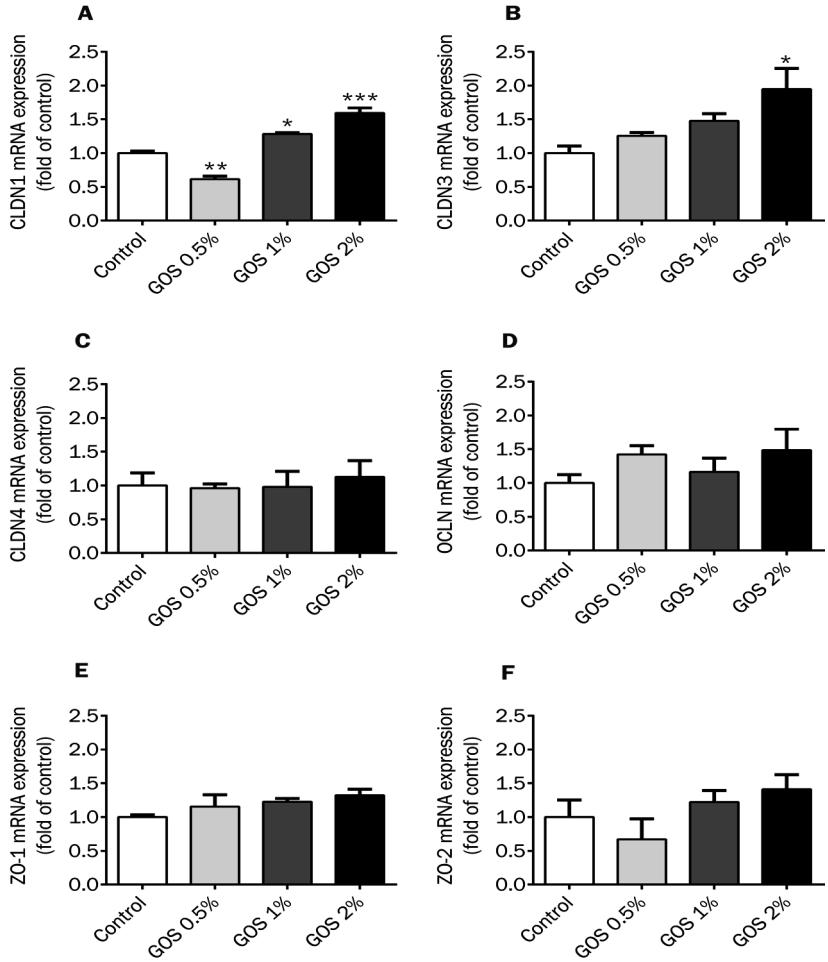

Figure 2. Effects of GOS on the mRNA expression of tight junction proteins. Caco-2 cells were grown on inserts with increasing concentrations GOS (0.5, 1 and 2%) on the apical and basolateral sides during 17 days. After 17 days of GOS incubation, mRNA expression levels of CLDN1 (A), CLDN3 (B), CLDN4 (C), OCLN (D), ZO-1 (E) and ZO-2 (F) were measured by qRT-PCR. Results are expressed as relative mRNA expression (fold of control, normalized to β-actin) as mean ± SEM of three independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001 significantly different from unstimulated cells).

Effects of GOS on the mRNA expression of AJ and desmosomes

Incubation of GOS for 17 days did not induce alterations in the mRNA expression of AJ proteins (E-cadherin, β-catenin) and desmosomes (DSP 1/2 and DSG2) (Figure 3). However, the mRNA expression levels of the desmosome, DSG2, were decreased by incubation with 2% GOS, but this was not significantly different from the control cells (Figure 3D).

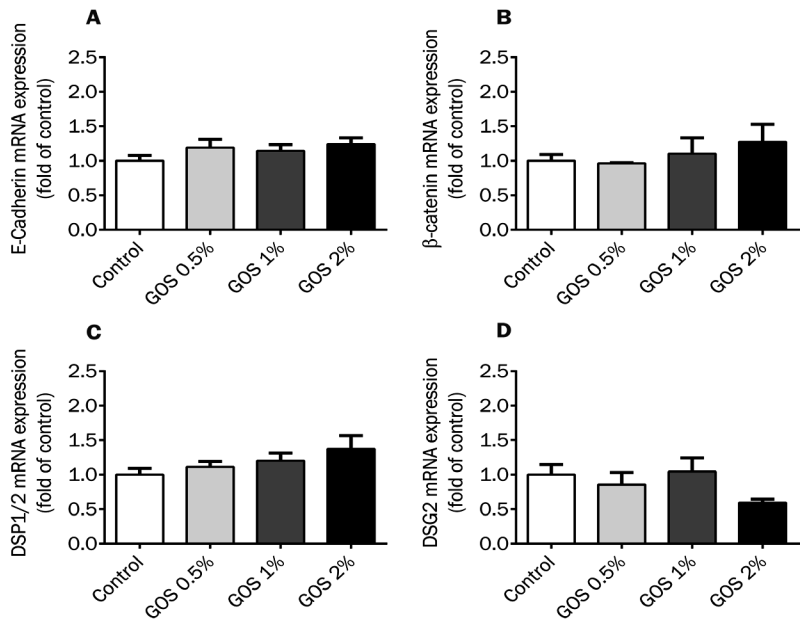

Figure 3. Effects of GOS on the mRNA expression of adherens junction and desmosomes proteins. Caco-2 cells were grown on inserts with increasing concentrations of GOS (0.5, 1 and 2%) on the apical and basolateral sides during 17 days. After 17 days of GOS incubation, mRNA expression levels of E-cadherin (A), β-catenin (B), DSP 1/2 (C) and DSG2 (D) were measured by qRT-PCR. Results are expressed as relative mRNA expression (fold of control, normalized to β-actin) as mean ± SEM of three independent experiments.

Effects of GOS on the protein expression of TJ proteins in Caco-2 cell monolayers

Western blot analyses showed that GOS incubation for 17 days did not significantly change the protein expression of CLDN1 (Figure 4A and 4C) and CLDN3 (Figure 4B and 4D).

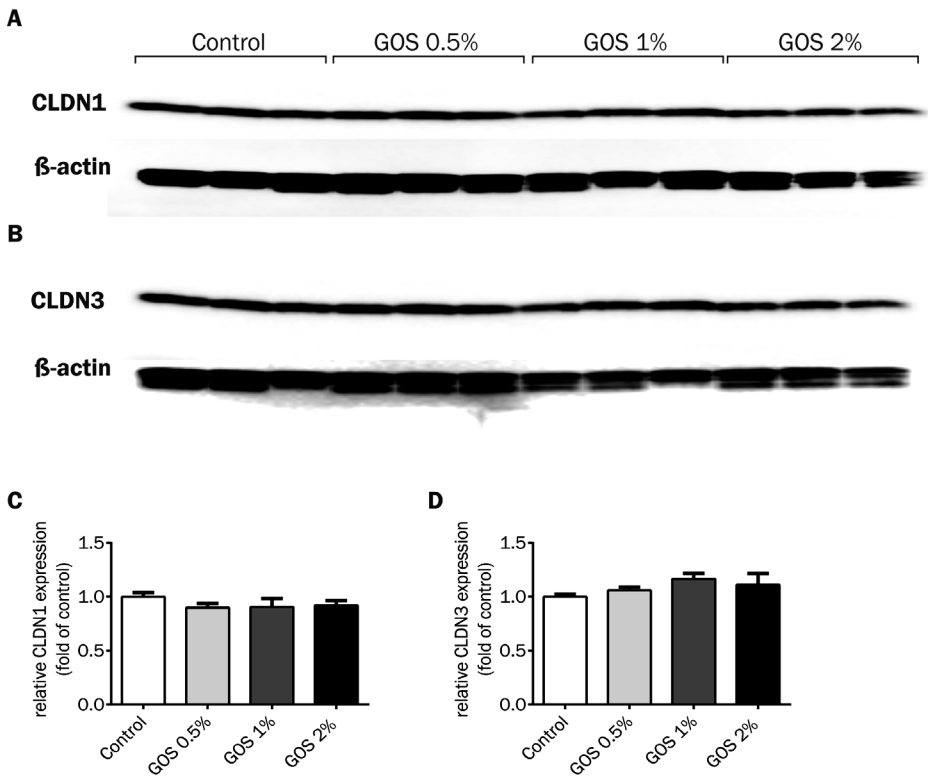

Figure 4. Effects of GOS on the protein expression of tight junction proteins in Caco-2 cell monolayers. Caco-2 cells were grown on inserts with increasing concentrations GOS (0.5, 1 and 2%) on the apical and basolateral sides during 17 days. After 17 days of GOS incubation, CLDN1 (A) and CLDN3 (B) protein levels were measured by Western blot analysis. Results are expressed as the relative CLDN1 (C) and CLDN3 (D) protein expression (fold of control, normalized to β-actin) as mean ± SEM of triplicates.

Effects of GOS on the cellular distribution of CLDN1 and CLDN3 in Caco-2 cell monolayers

In untreated cells, CLDN1 and CLDN3 are localized at the Caco-2 cell membrane and appear as continuous belt-like structures encircling the cells. In cells cultured with GOS the CLDN1 and CLDN3 expression seemed to be more pronounced compared to the control cells (Figure 5A and 5B).

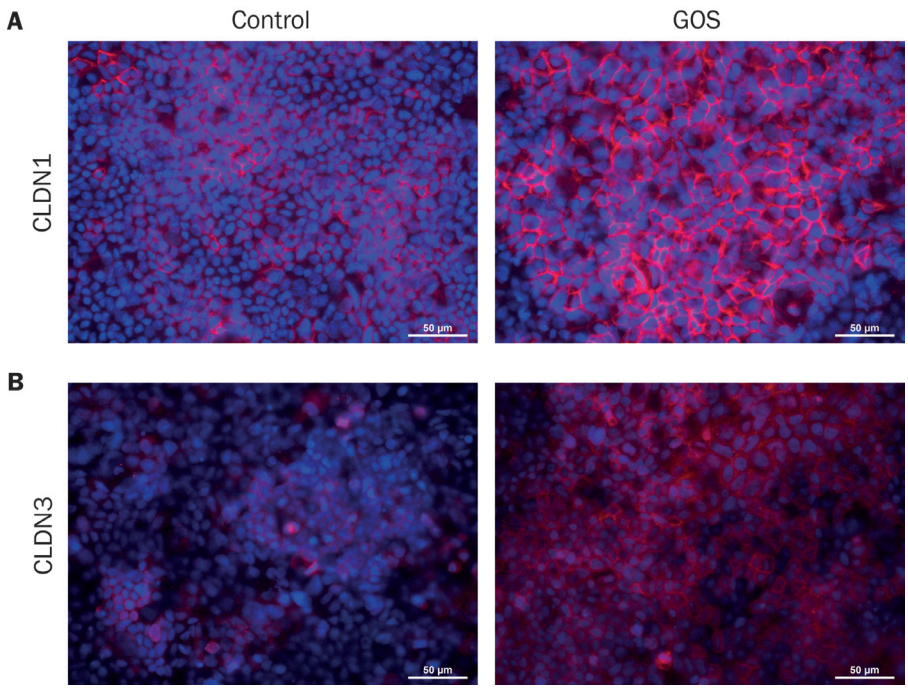

Figure 5. Effects of GOS on the cellular distribution of CLDN1 and CLDN3, in Caco-2 cell monolayers. Caco-2 cells were grown on inserts with and without 2% GOS on the apical and basolateral sides during 17 days. After 17 days of GOS incubation, cellular localization of CLDN1 (A) and CLDN3 (B) in the Caco-2 monolayers was assessed by an immunofluorescent staining (400x magnification). The scale bar represents 50 µm.

Effects of GOS on the cyclin D1 and Ki67 expression and distribution in Caco-2 cell monolayers

The mRNA expression levels of the proliferation markers, cyclin D1 and Ki67, were up-regulated in Caco-2 cells incubated with 2% GOS for 17 days compared to the unstimulated cells (Figure 6A and 6B). Western blot analyses showed that GOS incubation did not significantly change the cyclin D1 protein expression levels (Figure 6C and 6D).

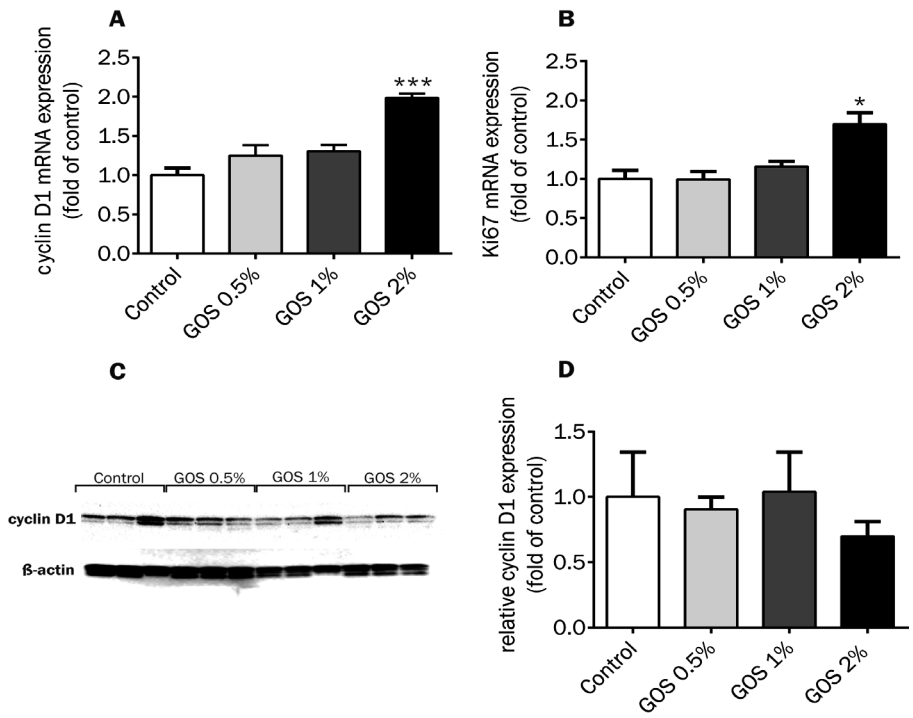

Figure 6. Effects of GOS on the cyclin D1 and Ki67 expression in Caco-2 cell monolayers. Caco-2 cells were grown on inserts with increasing concentrations GOS (0.5, 1 and 2%) on the apical and basolateral sides during 17 days. After 17 days of GOS incubation, mRNA expression levels of cyclin D1 (A) and Ki67 (B) were measured by qRT-PCR and cyclin D1 protein levels were measured by Western blot analysis (C, D). Results are expressed as relative mRNA or protein expression (fold of control, normalized to β-actin) as mean ± SEM of three independent experiments for mRNA analysis and three replicates for western blot analysis (*P < 0.05, ***P < 0.001 significantly different from unstimulated cells).

The immunofluorescence microscopy of Caco-2 cell monolayers displayed almost no Ki67- and cyclin D1-positive cells under control conditions, whereas the amount of Ki67- and cyclin D1-positive cells is slightly increased after 17 days GOS incubation (Figure 7A and 7B).

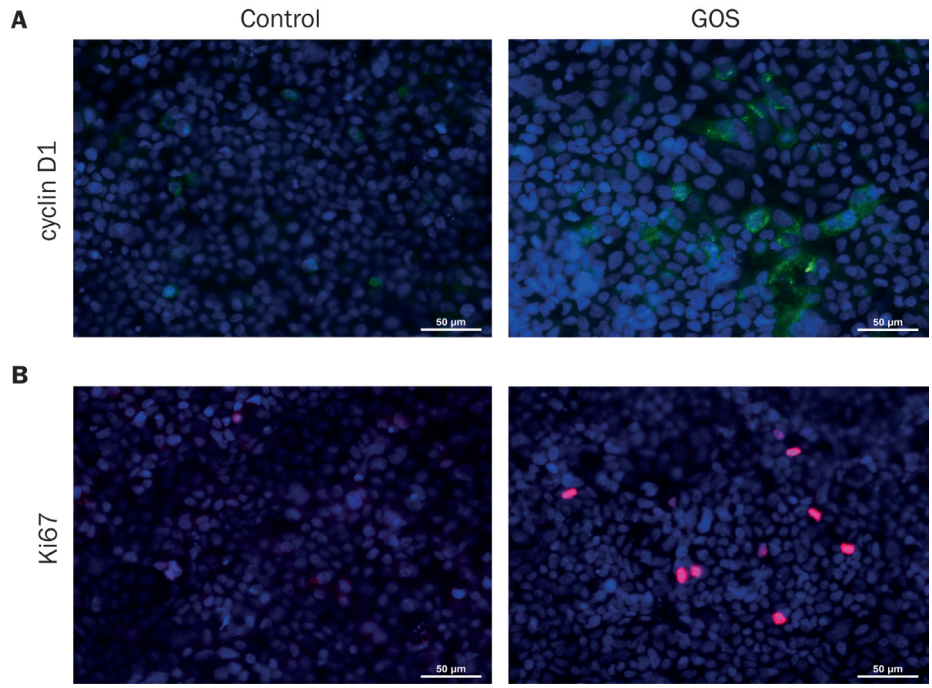

Figure 7. Effects of GOS on the cyclin D1 and Ki67 distribution in Caco-2 cell monolayers. Caco-2 cells were grown on inserts with and without 2% GOS on the apical and basolateral sides during 17 days. After 17 days of GOS incubation, cellular localization of cyclin D1 (green, A) and Ki67 (red, B) in the Caco-2 monolayers was assessed by an immunofluorescent staining (400x magnification). The scale bar represents 50 µm.

Discussion

In the current study we aimed to investigate the direct microbiota-independent effects of GOS on intestinal epithelial barrier development. We focused on the role of GOS during the epithelial maturation and differentiation phase. To this end, pre-confluent Caco-2 cells were used, which comprise the proliferation compartment of the crypt/villus axis [29]. Previously, different *in vitro* studies of our group focused on direct effects of GOS after short term exposure to confluent Caco-2 monolayers (24-48 h) [27,28], whereas limited information is available related to long term GOS exposure in pre-confluent Caco-2 cells. Several *in vitro* and *in vivo* studies indicated that HMO and prebiotic oligosaccharides, including GOS, are absorbed and transported across the epithelial barrier [36–38]. Therefore, in this study the Caco-2 cells monolayers were apically and basolaterally exposed to GOS-supplemented media.

Patel *et al.* [39] reported that commensal and probiotic bacteria play a key role in the developmental maturation of the intestinal barrier by regulating CLDN3 expression. Our data indicated that prebiotic treatment with GOS for 17 days can also accelerate both CLDN1 and CLDN3 mRNA expression levels and improve intestinal integrity (TEER) in an *in vitro* intestinal cell system lacking commensal bacteria. No alterations in the mRNA expression of AJ proteins (E-cadherin, β-catenin) and desmosomes (DSP 1/2 and DSG2) were observed after 17 days GOS incubation. Previously, we have demonstrated that neonatal piglets fed with a GOS-supplemented diet showed an up-regulation in mRNA levels of CLDN1 in different parts of the intestine after 3 or 26 days [26], which could be related to improved barrier integrity. Another study of our group revealed that GOS protect the intestinal barrier after a challenge with the mycotoxin deoxynivalenol (DON) in Caco-2 cells and B6C3F1 mice. Here, the expression and localization of CLDN3 and the acceleration of TJ re-assembly played an important role in the protective effect of GOS [27]. It is known that both CLDN1 and CLDN3 are barrier-forming claudins and can in some instances co-localize with ZO-1 at the junction, but are also expressed along the lateral cell membranes between adjacent epithelial cells to eliminate the extracellular space [30,40–43].

We might conclude that one mechanism by which GOS may induce an optimal epithelial barrier development is by inducing maturation of CLDN1/CLDN3-regulated intestinal epithelial permeability.

In addition, it should be noted that GOS is likely inducing other changes to the intestinal epithelium besides TJ barrier changes. In the current study, GOS also affected the epithelial cell proliferation, since the cyclin D1 and Ki67 mRNA expression levels were up-regulated in GOS-stimulated cells. Holscher *et al.* [24] observed that different HMO inhibited proliferation in undifferentiated intestinal epithelial cells (HT-29 and Caco-2Bbe cells) after 72 h incubation. However, in contrast to our study, these cells were exposed to HMO for a short exposure period during cell differentiation and maturation. We also observed a decrease in the amount of GOS-stimulated cells compared to

unstimulated cells within the first 3 days of exposure (data not shown). Functional analyses of claudins in cell cultures and in mice have suggested that claudin-based TJs may have pivotal functions in the regulation of the epithelial microenvironment (e.g. ionic conditions), which is critical for cell proliferation [44,45]. TJs can recruit signaling proteins that participate in the regulation of cell proliferation and differentiation [46]. It has been demonstrated that changes in the expression of the claudin family may lead to increased cell growth, however the (in)direct mechanisms linking claudin proteins to cell proliferation are not clear yet [44,47]. For the ZO family proteins, it is reviewed by Farkas *et al.* [47] that these proteins can directly interact with proliferative processes via the regulation of cyclin D1 localization, protein stability and gene transcription. The ZO family of TJ plaque proteins can sequester cyclin D1 at TJs during mitosis which leads to cell-cycle progression and cell proliferation [48]. However, in the current study the ZO-1 mRNA expression was not increased after 17 days of GOS incubation.

Previous investigations with other oligosaccharides (FOS and xylo-oligosaccharides) speculated that an increased proliferation rate of epithelial cells in the colon of neonatal piglets and rats is related to the short-chain fatty acid production [49,50]. The current study confirmed that the prebiotic-induced increase in epithelial proliferation might be partly regulated via microbiota-independent functions.

Bhatia *et al.* [10] observed a direct effect of GOS in a colonic adenocarcinoma (LS174T) cell line on the goblet cell secretory products, such as trefoil factor 3 (TFF3) which plays a pivotal role in epithelial restitution, mucosal protection and enhancement of structural integrity in mucosal barrier. It might be that the improved intestinal TJ barrier integrity is related to toll-like receptor 2 (TLR2) signaling via a mechanism involving TFF3 and the activation of the PI3K/Akt pathway [51]. However, also other microbiota-independent pathways are possible, including interaction with other TLRs, carbohydrate receptors, inductions of galectins and anti-oxidative responses [11,12,28]. To unravel the microbiota-independent mechanism of action of GOS and other non-digestible oligosaccharides, further studies are warranted.

Conclusion

Taken together, the current experiments provide significant evidence for microbiota-independent effects of GOS on th intestinal epithelial cell integrity and epithelial proliferation contributing to the development of the intestinal barrier in early phases of life and/or strengthen the epithelial barrier in later phases of life by encountering luminal antigens, pathogens and toxins.

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Chapter 8

Mitogen-activated protein kinases as a possible target for galacto-oligosaccharides: preliminary results

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Abstract

Mitogen-activated protein kinase (MAPK) signal transduction pathways are among the most important mechanisms for homeostatic and regulatory responses in eukaryotic cells, including differentiation, cell movement, cell division, and cell death. Previously, we provided evidence for direct, microbiota-independent, effects of galacto-oligosaccharides on intestinal epithelial cells related to intestinal barrier integrity and inflammatory responses. The mode of action of GOS is complex and still not entirely understood, hence this study started to unravel the possible mechanism of action of GOS in relation to MAPKs (c-Jun NH2-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK)) using an *in vitro* model with intestinal epithelial Caco-2 cells. Caco-2 cells were pre-incubated with GOS in presence or absence of MAPK inhibitors for 24 h followed by calcium deprivation and transepithelial electrical resistance was measured during calcium recovery. The paracellular tracer flux of Lucifer yellow and the protein expression of different adherens and tight junction proteins, including epithelial cadherin (E-cadherin), zona occludens protein-1 (ZO-1) and occludin (OCLN), were measured after 8 h recovery.

The ERK- and JNK-inhibitor significantly inhibited the GOS-induced acceleration in barrier reassembly. The slight increase in E-cadherin protein expression by GOS 8 h after calcium deprivation was significantly prevented by the ERK inhibitor.

In a separate experimental set-up, Caco-2 cells were pre-incubated with GOS for 24 h prior to a challenge with the common food/feed contaminant, the mycotoxin deoxynivalenol (DON), for 4 h and 24 h. Pretreatment with GOS attenuated the DON-induced phosphorylation of p38 MAPK.

These preliminary results indicate that there is a possible relationship between the microbiota-independent effects of GOS on intestinal epithelial cells and MAPK signaling, although this should be further elucidated.

Introduction

Protein kinases are enzymes that covalently attach phosphate to the side chain of target proteins inside cells and this phosphorylation process can control different functions of proteins [1]. Mitogen-activated protein kinase (MAPK) signal transduction pathways are among the most important mechanisms for homeostatic and regulatory responses in eukaryotic cells, including differentiation, cell movement, cell division, and cell death [2,3]. Three well characterized subfamilies of MAPKs control a vast array of physiological processes: the extracellular signal-regulated kinases (ERK), which preferentially regulates cell growth and differentiation, the p38 MAPK and the c-Jun NH2-terminal kinases (JNK) which function mainly in stress responses, including inflammation and apoptosis [4].

It has also been demonstrated that MAPKs play a major role in regulation of intestinal barrier function. Different studies described that intestinal barrier dysfunction induced by different stressors is related to activation of MAPK [5–8].

Our group provided evidence for a protective effect of non-digestible oligosaccharides, including galacto-oligosaccharides (GOS), on intestinal barrier function. GOS protected the tight junction network and accelerated tight junction (TJ) reassembly via direct interaction with intestinal epithelial cells [9–11]. Furthermore, heat stress-induced disruption of the intestinal epithelial barrier was mitigated by GOS especially by modulating E-cadherin expression and by suppressing the heat-induced oxidative stress [11]. Laprise *et al.* [12] described the possible role of E-cadherin and concomitant ERK signaling in proliferation/differentiation transition along human intestinal epithelium, whereas activation of multiple MAPK signal transduction pathways occurred during oxidative stress–induced intestinal epithelial cell injury [13,14].

Moreover, GOS prevented the inflammatory response of intestinal epithelial cells in response to the common food and feed contaminant, the mycotoxin deoxynivalenol (DON), as measured by the CXCL8 expression and secretion [9]. It has been reported that the immunomodulatory effects of DON are related to MAPK pathways [15,16] and particularly p38 MAPK mediates CXCL-8 induction [17]. The DON-induced gene upregulation at transcriptional and post-transcriptional level is also associated with MAPKs (as reviewed by Pestka [15]).

Considering all these direct, microbiota-independent, effects of GOS on intestinal epithelial cells and the conceivable relation with MAPK signal transduction pathways, this study started to unravel the possible mechanism of action of GOS in relation to MAPKs using an *in vitro* model of intestinal epithelial Caco-2 cells that were either stressed by calcium deprivation or by exposure to the mycotoxin DON.

Materials and Methods

Cell culture

Human epithelial colorectal adenocarcinoma (Caco-2) cells were obtained from the American Type Tissue Collection (Code HTB-37) (Manassas, VA, USA, passage 79-91) and were cultured as described previously [18]. Briefly, cells were seeded in transwell inserts placed in a 24-well plate and were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C and refreshed every 2-3 days during 17 days.

GOS

The commercially available Vivinal® GOS syrup (FrieslandCampina Domo, Borculo, The Netherlands), comprising 45% GOS with 2-8 saccharide units (degree of polymerization) 16% free lactose, 14% glucose, and 25% water, was used. The GOS cytotoxicity was evaluated previously showing no cytotoxicity induced by 2% GOS in Caco-2 cells [9].

MAPK inhibitor study

The different MAPK inhibitors, c-Jun N-terminal kinase (p-SAPK/JNK) inhibitor (SP600125, Sigma, St Luis, Mo, USA), extracellular signal-regulated kinase (ERK, p44/42) inhibitor (PD98,059, Sigma, St Luis, Mo, USA) and p38 mitogen-activated protein kinase (p38) inhibitor (SB 203580, Sigma, St Luis, Mo, USA) were dissolved in DMSO (50 mM stock solution). Solutions of the JNK inhibitor (50 μM), ERK inhibitor (10 and 50 μM) and p38 inhibitor (10 μM) were prepared by dissolving the stock solutions either in DMEM or in DMEM supplemented with 2% GOS. Caco-2 cells were pre-incubated with DMEM or DMEM supplemented with 2% GOS in presence or absence of different MAPK inhibitors added to the apical and basolateral sides for 24 h prior to the calcium switch assay.

Calcium switch assay

The calcium switch assay was performed as described previously [9]. Briefly, Caco-2 cells were deprived from calcium by exposure to 2 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (Sigma, St Luis, Mo, USA) in calcium and magnesium free Hanks' Balanced Salt Solution (HBSS) (Gibco, Invitrogen, Carlsbad, CA, USA) for 20 minutes. Subsequently, cells were rinsed and allowed to recover in either DMEM (containing 2mM $CaCl_2$) or in DMEM supplemented with 2% GOS in presence or absence of different MAPK inhibitors (JNK (50 μM), ERK (50 μM), p38 (10 μM)). During the recovery period, the transepithelial electrical resistance (TEER) was determined at different time points (2 h, 4 h, 6 h, and 18 h).

The same protocol was followed for two different concentrations (10 and 50 μM) of the ERK inhibitor, but the TEER was measured at 20 minutes, 4 h and 8 h after the calcium switch, the paracellular tracer flux assay was conducted and the cell lysates obtained at the final time point (8 h) were preserved for epithelial cadherin (E-cadherin), zona occludens protein-1 (ZO-1) and occludin (OCLN) Western blot analyses.

Paracellular tracer flux assay

The paracellular tracer flux was measured with the use of Lucifer yellow (LY, molecular mass: 0.457 kDa, Sigma, St Luis, Mo, USA), which was added to the apical side of the transwells after 4 h recovery (calcium switch assay). After 4 h incubation (8 h recovery calcium switch assay) the amount of LY at the basolateral side 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, respectively.

Western blot analysis

Caco-2 cells were lysed using 50 µl RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0) (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany) and were harvested after centrifugation (14.000 g for 15 minutes). Total protein concentrations were obtained by the BCA protein assay according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, USA). Normalized samples were boiled and separated by electrophoresis (Criterion™ Gel, 4–20% Tris– HCl, Bio-Rad, Hercules, CA, USA) and proteins were electrotransferred onto polyvinylidene difluoridemembrane (Bio-Rad, Veenendaal, The Netherlands). Membranes were blocked with PBS containing 0.05% Tween-20 (PBST) and 5% milk proteins for 1 h at room temperature. Membranes were incubated with the primary antibodies detecting phospho-p44/42 and phospo-p38 MAPKs (1:1000, Cell Signaling, Danvers, MA, USA), OCLN, ZO-1 (1:1000, Invitrogen, Carlsbad, CA, USA) and E-cadherin (1:1000, eBioscience, San Diego, CA, USA) at 4˚C, overnight. After washing with PBST, membranes were incubated with appropriate horseradish peroxidase conjugated secondary antibodies (1:2000, Dako, Glostrup, Denmark) for 2 h at room temperature. In order to assess the equality of loading membranes were probed with β-actin antibody (1:4000, Cell Signaling, Danvers, MA, USA). Finally, membranes were incubated with ECL Prime western blotting and visualized as described previously [19].

***In vitro* studies with DON and GOS**

The mycotoxin DON was used as relevant model compound for the disruption of the barrier integrity of the Caco-2 monolayer. DON (D0156; Sigma-Aldrich, St Luis, Mo, USA) was dissolved in absolute ethanol (99.9%, JT Baker, Deventer, The Netherlands) and stored at -20˚C. Caco-2 cells were grown on inserts and were pre-incubated with 2% GOS (apical and basolateral compartments) for 24 h followed by 4.2 µM DON stimulation (apical and basolateral compartments) for 4 and 24 h. The Caco-2 cell lysates were preserved for western blot analyses of phosphorylated MAPK.

Statistical analysis

Experimental results were expressed as mean ± SEM. 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 ANOVA with Bonferroni post-hoc test. Results were considered statistically significant when $P < 0.05$.

Results

Effects of different MAPK inhibitors on GOS-induced acceleration of TJ reassembly after calcium deprivation in Caco-2 cells

The ERK, JNK and p38 inhibitors did not affect the TJ reassembly in untreated cells 2, 4 and 6 h after calcium deprivation as assessed by TEER measurements. After 18 h recovery, inhibition of ERK resulted in a significant reduction of TJ reassembly (Figure 1A).

Pretreatment with 2% GOS accelerated the TJ reassembly time-dependently during a period of 18 h and the first significant effect of GOS was observed after 4 h of calcium recovery (Figure 1A). The GOS-induced acceleration in TJ reassembly after calcium deprivation was significantly inhibited by 50 μM ERK and 50 μM JNK inhibitors, but not by the p38 inhibitor (10 μM) after 4, 6 and 18 h recovery (Figure 1A).

Effects of ERK inhibitor on GOS-induced acceleration of TJ reassembly and LY translocation in Caco-2 cells

To investigate the role of ERK in the GOS-induced acceleration of TJ reassembly in more detail, different concentrations of the ERK inhibitor were used. Although both concentrations of the ERK inhibitor (10 and 50 μM) significantly inhibited the TEER increase after 4 h of calcium recovery in GOS-stimulated cells, GOS did not significantly accelerate the TJ reassembly 4 h after recovery. The GOS-induced significant restoration in TEER values was totally inhibited by the ERK inhibitor at a concentration of 50 μM after 8 h calcium recovery (Figure 1B). After 4 h as well as 8 h calcium recovery, the ERK inhibitors (10 and 50 μM) significantly reduced the TJ reassembly under control conditions (Figure 1B). This reduction in TJ reassembly was confirmed by a dose-dependent increase in LY translocation across the untreated Caco-2 cell monolayer induced by the ERK inhibitors after 8 h calcium recovery (Figure 1C). Although the ERK inhibitor (50 μM) significantly prevented TJ reassembly observed by an increase in LY translocation in GOS-stimulated cells, GOS did not significantly affect the LY translocation across the Caco-2monolayer(Figure 1C).

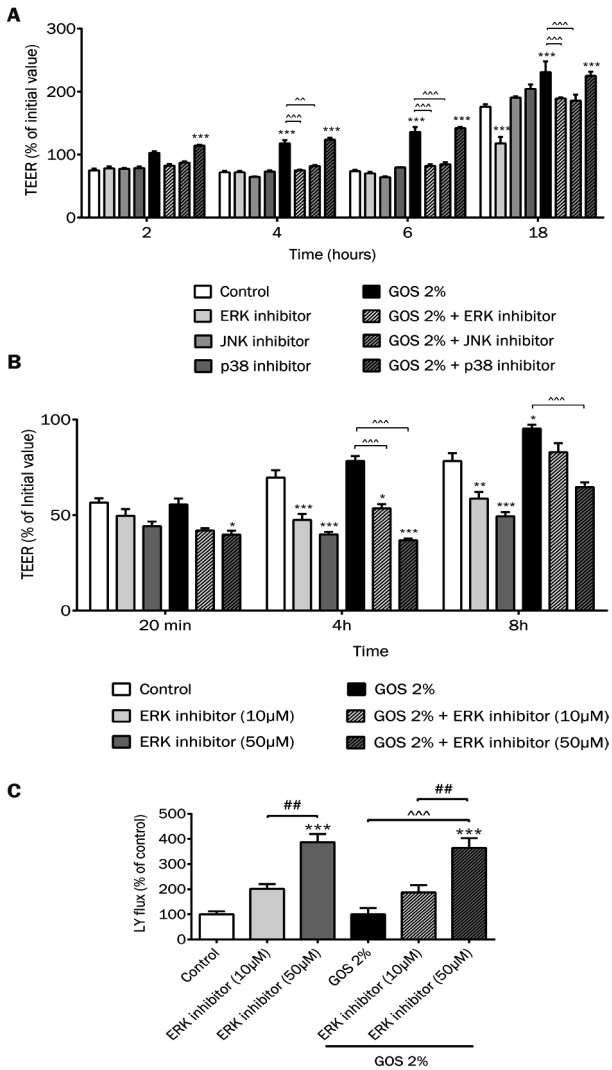

Figure 1. Caco-2 cell monolayers were pre-treated apically and basolaterally (24 h) with DMEM or DMEM supplemented with 2% GOS in presence or absence of 10 or 50 μM ERK inhibitor (A, B, C), 50 μM JNK inhibitor (A), 10 μM p38 inhibitor (A) prior to calcium deprivation. The TEER was measured at indicated time points (A and B) during recovery. LY transport from apical to basolateral side was measured after 8 h calcium recovery (C). Results are expressed as a percentage of initial value (TEER) or as a percentage of control cells (LY flux) as means ± SEM of three replicates/condition (A) or three independent experiments (B, C). *P < 0.05 **P < 0.01, ***P < 0.001 significantly different from untreated cells; ^^P < 0.01, ^^^P < 0.001 significantly different from GOS-treated cells; ##P < 0.01 significantly different from control or GOS-treated cells in presence of ERK inhibitor (10 μM).

Effect of ERK inhibitor on protein expression of E-cadherin, ZO-1 and OCLN

Pretreatment with 2% GOS showed a slight increase in the E-cadherin protein expression in a Caco-2 cell monolayer 8 h after calcium deprivation, whereas the protein expression of ZO-1 and OCLN was not affected by 2% GOS (Figure 2). The ERK inhibitor significantly prevented the possible GOS-induced enhancement of E-cadherin protein expression 8 h after calcium deprivation (Figure 2).

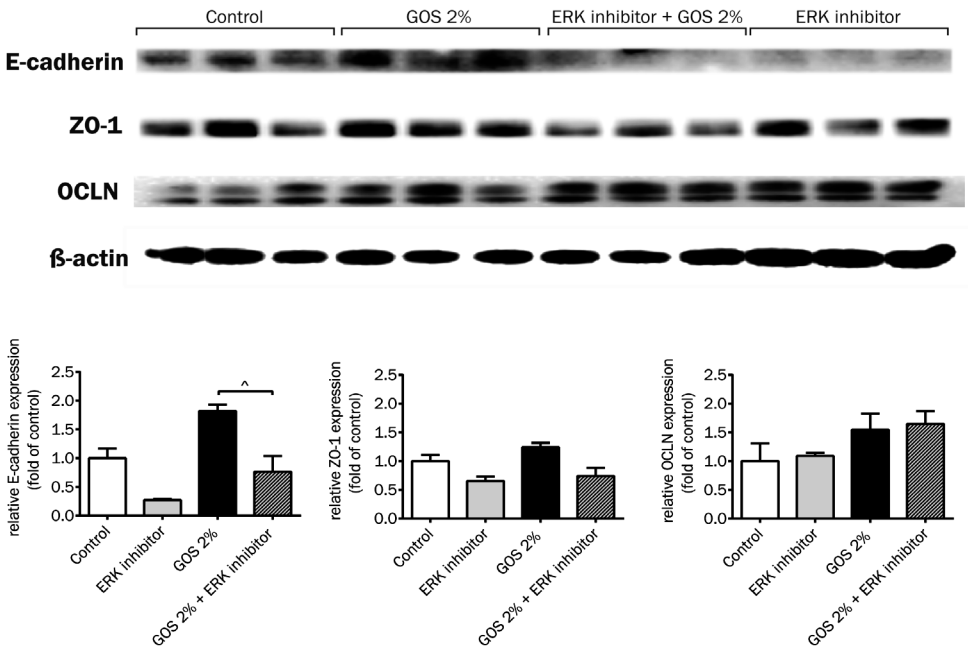

Figure 2. Caco-2 cell monolayers were pre-treated apically and basolaterally (24 h) with DMEM or DMEM supplemented with 2% GOS in presence or absence of 50 μM ERK inhibitor prior to calcium deprivation. After 8 h calcium recovery, protein expression levels of E-cadherin, ZO-1 and OCLN were measured by Western blot analysis. Results are expressed as relative protein expression (fold of control, normalized to β-actin) as means ± SEM of three replicates/condition. ^P < 0.05 significantly different from GOS-treated cells.

Effects of DON and GOS stimulation on the phosphorylated ERK, JNK and p38 MAPK in Caco-2 cells

Exposure of Caco-2 cells to DON, induced a clear upregulation of phosphorylated p38 protein expression after 4 and 24 h (Figure 3). After 4 h DON stimulation an increased expression of the phosphorylated ERK protein was observed that returned towards basal levels after 24 h (Figure 4). There was no effect of 2% GOS on the phosphorylated p38 and ERK expression (Figure 3 and Figure 4). DON and 2% GOS did not induce phosphorylation of JNK protein expression in Caco-2 cells (data not shown).

DON-induced phosphorylated p38 was not changed by 24 h GOS pre-incubation at the 4 h time point, whereas after 24 h of DON stimulation, p38 phosphorylation was moderated by GOS (Figure 3). In addition, the increased phosphorylation of ERK induced by DON was not affected by GOS pre-incubation after 4 h DON exposure (Figure 4).

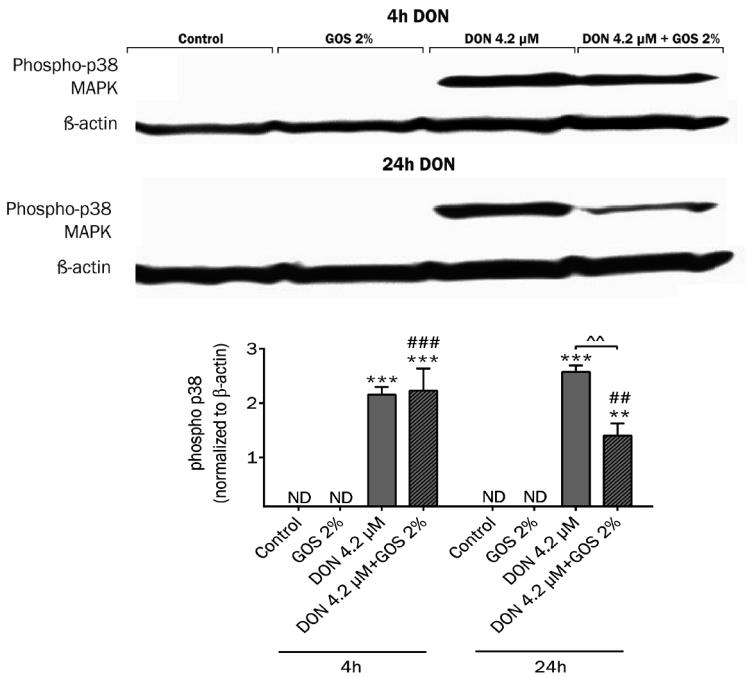

Figure 3. Caco-2 cell monolayers were pre-treated apically and basolaterally (24 h) with DMEM or DMEM supplemented with 2% GOS followed by DON stimulation for 4 and 24 h. Protein expression levels of phosphorylated-p38 were measured by Western blot analysis. Results are expressed as phosphorylated protein expression (normalized to β-actin) as means ± SEM of three independent experiments. *P < 0.05, ** P < 0.01, ***P < 0.001; significantly different from control group; ^^P < 0.01; significantly different from DON group; ##P < 0.01, ###P < 0.001; significantly different from GOS group.

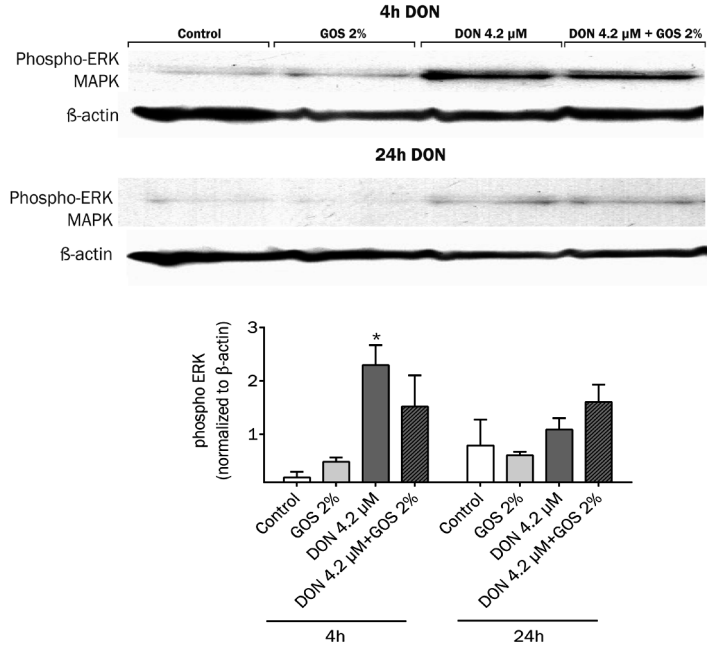

Figure 4. Caco-2 cell monolayers were pre-treated apically and basolaterally (24 h) with DMEM or DMEM supplemented with 2% GOS followed by DON stimulation for 4 and 24 h. Protein expression levels of phosphorylated-ERK were measured by Western blot analysis. Results are expressed as phosphorylated protein expression (normalized to β-actin) as means ± SEM of three independent experiments. *P < 0.05; significantly different from control group.

Discussion

In the current study the role of MAPK signaling in the direct and microbiota-independent GOS effect on intestinal epithelial cells is investigated. First, we showed that MAPK ERK is important for spontaneous TEER recovery after calcium deprivation and the related paracellular tracer transport was increased after inhibition of ERK signaling. The regulatory function of the ERK MAPK cascade (also known as Ras-Raf-MEK-ERK pathway) has been demonstrated in intestinal cell proliferation, since inactivation of this ERK MAPK pathway is necessary for the terminal differentiation process of intestinal epithelial cells [20]. Different mechanisms are involved in ERK-mediated intestinal cell differentiation and integrity. It has been shown that MAPK ERK kinase (MEK) and its downstream effectors are required for assembly of TJs in intestinal epithelial cells, especially for claudin-dependent assembly [21].

It has also been described that under-differentiated and differentiated intestinal epithelial cells (Caco-2) can respond differently to the ERK signaling pathway. Knockdown of ERK enhanced TJ integrity and accelerated TJs assembly in under-differentiated Caco-2 cells, whereas in differentiated Caco-2 cells the ERK1/2 knockdown resulted in a delay in TJs assembly [22]. The latter observation might be a good explanation for the results in the current study, since GOS accelerated the TJ reassembly of differentiated Caco-2 cells after calcium deprivation and this GOS-induced acceleration was decreased by inhibiting the ERK signaling pathway. Although GOS did not significantly affect the LY translocation 8 h after calcium deprivation, the ERK inhibitor significantly induced an increase in LY translocation in GOS-stimulated cells. The effects of GOS on TEER recovery were not confirmed by the LY paracellular permeability.

In addition, the ERK inhibitor significantly prevented the GOS-induced enhancement of the E-cadherin protein expression 8 h after calcium deprivation, whereas the protein expression of ZO-1 and OCLN was not significantly altered by 2% GOS and/or the ERK inhibitor. Laprise *et al.* [12] provided evidence that one of the earliest molecular events resulting from the engagement of E-cadherin in areas of cell–cell contact in epithelial cells is the inhibition of the MEK/ERK cascade.

In conclusion, our results suggest that ERK might serve as a critical player in the GOS-induced acceleration in TJ reassembly in differentiated epithelial cells. However, future research should not only focus on the ERK signaling pathway, since JNK might also play a role in the GOS-induced TEER recovery in intestinal epithelial cells as observed in the current study.

It is known that the common food and feed contaminant, the mycotoxin DON, as a transcriptional inhibitor can induce MAPK-driven chemokine and cytokine production via a mechanism called the ribotoxic stress response [23]. It has been observed that p38 MAPK play an important role in DON-induced CXCL8 expression [17]. Results from this study confirm the DON-induced phosphorylation of p38 in Caco-2 cells 4 and 24 h after stimulation. Both p38 MAPK and nuclear factor (NF)-kappa B have been recognized to

play a role in the DON-induced CXCL8 expression [17,24]. A study by Hippenstiel *et al.* [25] showed that p38 activation can be linked to NF-κB activation, evidenced by the regulatory function of Rho-signaling pathway and p38 kinase cascade in LPS-induced NF-κB activation and CXCL-8 synthesis in human endothelial cells.

Our group already described that GOS prevented the DON-induced CXCL8 expression and release in Caco-2 cells [9]. Here, pretreatment with 2% GOS prior to 24 h DON exposure showed a significant decrease in the DON-induced phosphorylated p38 MAPK protein expression, whereas the DON-induced phosphorylated ERK MAPK protein expression was not affected by GOS-pretreatment. The phosphorylated JNK MAPK was neither detectable after DON stimulation nor after GOS pretreatment. Different observations of DON-induced activation of MAPKs, including ERK, JNK and p38 have been described in human as well as porcine intestinal cell lines. DON-activated MAPKs could lead to an impaired homeostasis of intestinal tissue and a reduction in barrier function as well as a decrease in the expression of claudin4 [6,26]. In addition, Capitán-Cañadas *et al.* [27] already described that the mechanism of action of oligosaccharides might be related to MAPKs, since elevated cytokine levels induced by oligosaccharides (e.g. FOS and inulin) in rat primary monocytes could be suppressed by MAPKs inhibitors.

Future studies should focus on examination of the ratios of non-phosphorylated *versus* phosphorylated MAPK proteins in Caco-2 cells exposed to DON and/or GOS.

Taken together, these preliminary results provide evidence that there is a possible link between the direct effects of GOS on intestinal epithelial cells and MAPK signaling, although further research is needed to unravel the exact role of MAPK in this process.

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Chapter 9

General discussion

General discussion

Mycotoxins: major food contaminants

Food and feed consumption serves not only as a source of nutrients, but may expose humans and animals also to a broad variety of potentially harmful substances [1–3]. The most prevalent natural toxins in food are mycotoxins, plant secondary metabolites, bacterial toxins, and phycotoxins [4–6]. Mycotoxins, representing a chemically very diverse class of fungal toxins, have been associated with acute organ toxicity, such as hepatotoxic, pulmotoxic, cardiotoxic and nephrotoxic effects, as well as reproductive toxicity, genotoxicity and carcinogenesis. More recently, immunomodulatory effects induced by mycotoxins in humans and animals gain increasing attention. The most prevalent mycotoxins of human and animal health concern are aflatoxins, fumonisins, ochratoxins, zearalenone derivatives and the group of trichothecenes [4,7,8].

The mechanism of toxicity of mycotoxins, are determined by their chemical structure. Due to the broad diversity of chemical structures among the class of mycotoxins, biochemical effects at the cellular level encompass interactions with cell membranes, interference with the cellular energy metabolism, interactions with macromolecules and the inhibition of DNA and protein synthesis [4,5,9–11]. Based on the mode of action as well as the toxicokinetic behaviour, significant differences in the sensitivity of individual species, gender and age groups are observed.

Considering the frequent contamination of food and feed commodities and the impossibility to prevent mycotoxin contamination at the pre-harvest stage, various intervention strategies to prevent the adverse effects induced by mycotoxins have been explored [12,13] (for details see **Chapter 2**). These include the application of organic and inorganic entero-adsorbents as well as the use of microorganisms, such as yeasts and bacteria, as well as fungal and bacterial enzymes that are able to modulate the structure of individual mycotoxins, rendering less or even non-toxic breakdown products [12,14]. However, reviewing the available literature, it has to be concluded that none of the currently available methods is able to be applicable for the inactivation of all mycotoxins and particularly deoxynivalenol (DON) has been identified as one of the toxins awaiting new intervention strategies.

Deoxynivalenol: a globally emerging fusariotoxin

In the last decennium an increasing prevalence of contamination with trichothecenes, specifically DON with a characteristic epoxide group at position 12-13, is observed in cereals and grains, resulting in a frequent exposure of humans and farm animals [11,15–19]. Wheat and maize were reported as the most frequent sources for DON contamination in Europe evidenced by 61% wheat and 89% maize positive tested samples [17]. A three year survey between 2009 and 2011 showed that DON was present in 59% of tested commodities, that mostly were used for feed production, which were sourced in the North and South America, Europe and Asia [16]. In an additional

large scale study DON has been detected in 57% of the tested food samples, as the most prevalent *Fusarium* mycotoxin in Europe [18]. DON exposure was confirmed by urine analysis and was detected in 98.68%, 90% and 100% of tested urine samples of adults from France, Sweden and United Kingdom, respectively [20–22]. A recent study in Belgium, showed all tested urine samples from adults to be positive for the human DON metabolite, DON-15-glucuronide, whereas the other human metabolite, DON-3-glucuronide, was quantified in 77% of the samples [23]. In this study, the prevalence of positive samples in children was even higher amounting to 100% for the DON-15-glucuronide.

This unexpected high exposure rate, as evidenced by measuring urinary DON excretion as a biomarker of exposure, can be attributed to a variety of factors, such as agricultural practice, changes in fungal genetics, as well as climate change. An additional finding was that DON occurs not only in its parent form, but also in several modified forms related to fungal and plant metabolism. Toxicogenic fusaria produce not only DON, but also 3- and 15-acetyl-DON (3ADON and 15ADON) [24,25] that contribute to the overall exposure, as the glucuronides have also been detected in human urine samples as indicated above.

Moreover, recent evidence suggests that DON is also metabolized by plants following the invasion by toxicogenic fusaria, yielding different conjugated forms (e.g. DON3-glucoside, DON3G), often denoted as “masked mycotoxins”. Plants conjugate the fungal metabolites to one or more sugar molecules and this reaction changes the physico-chemically properties of DON considerably. The glucose-conjugates are not extracted by commonly used extraction methods (hence the name “masked mycotoxins”) and are not detectable by conventional analytical techniques. This has led to an underestimation of the total mycotoxin-concentration in cereal products in the past. The glucose DON conjugates, particularly the DON3G, have been reported to occur in wheat, maize, oat, barley, malt and beer (for review see ref. [6]). Following ingestion of food, DON3G is hydrolysed by the intestinal microbiota resulting in the liberation of the parent toxin DON [26]. Subsequently, liberated DON is further converted into deepoxy-deoxynivalenol (DOM-1) by the intestinal microbiota via hydrolysis of the epoxide moiety in the position 12 and 13 of the molecular structure [10], which results in a reduction of the biological activity of DON [27]. Although DOM-1 is formed by intestinal bacteria, only a small percentage of the ingested DON was detected in human urine as DOM-1 [26], which might be associated with a limited rate of conversion by human microbiota and/or a limited absorption of DOM-1 from the gut lumen [10].

Considering the high absorption rate of DON in the upper parts of small intestine in most animal species as well as humans [10,28] and the stability of DON3G in upper small intestines due to the low bacterial counts, the release of DON from DON3G cleavage will mainly contribute to exposure of epithelial cells in the distal segments of intestine [29]. Hence, the observed vulnerability of the distal intestine may be related to the mammalian kinetics of DON.

A more detailed description of the complex kinetic behaviour of DON is presented in **Chapter 3**, together with the toxicological effects of these various fungal, plant and mammalian metabolites in the Caco-2 cell model. It can be suggested that the combined exposure of humans and animals to DON and its common modified forms needs to be considered in the risk characterization of DON.

The intestinal tract: target organ for DON

DON induces a broad range of adverse effects in animals and humans such as vomiting, gastroenteritis, protein and nucleic acid synthesis inhibition and apoptosis [11,19]. Moreover, DON has been identified as one of the toxins, targeting specifically the intestinal barrier integrity [2,10,30]. The intestinal barrier covers a surface of about 400 m^2 with specialized epithelial cells, which constitute a barrier separating the internal body tissues from the external environment [3,31]. This barrier serves two opposing and important functions for the maintenance of its functionality: 1. it allows nutrient absorption and 2. it limits the penetration of hazardous substances, such as antigens, toxins and allergens [3,32]. Any alteration in the functionality of the intestinal barrier is associated with the risk that allergens and pathogens pass the barrier, initiate an inflammatory reaction and contribute to the development of chronic diseases, such as (food) allergies and chronic inflammatory bowel diseases (IBD) [1,3,33,34]. This selective barrier function is achieved by a consistent line of epithelial cells, connected by a complex protein network, including tight junctions (TJs), adherens junctions and desmosomes, which link adjacent cells and seal the space between individual cells [35,36]. In a series of publications our group recently investigated in more detail the effects of DON on the intestinal barrier showing that one of the major targets of DON are the TJs [37–39].

These previous studies are completed with data from **Chapter 3**. In consideration of the findings that DON occurs also in various modified forms, we conducted a comparative study in the established Caco-2 cell model, testing the effect of DON, its acetylated fungal metabolites, as well as the plant-derives glucoside and the bacterial degradation product DOM-1. As expected, DOM-1 and DON3G showed no significant toxicological effects, whereas 3ADON was less potent to impair the intestinal barrier integrity compared to DON and 15ADON tended to be more potent to induce adverse effects on barrier integrity. Moreover, we demonstrated that non-digestible galacto-oligosaccharides (GOS) protect the intestinal epithelial barrier against DON and its acetylated derivatives (3ADON and 15ADON) in a concentration-dependent manner (**Chapter 3**).

The intestinal effects of DON in piglets

Previous *in vivo* experiments focussed particularly on the effects of DON at higher doses, showing a variety of acute toxic effects, such as vomiting and reduced feed intake and weight gain. Considering the increasing evidence that the intestines are the first target of DON following ingestion of contaminated feed, we designed a specific experiment

with 6-week-old piglets (the most sensitive animal species) to investigate early intestinal effects that may occur already at low feed contamination rates, which do not cause any visible clinical signs of intoxication (**Chapter 4**). The choice of the low DON dose (0.9 mg/kg feed) was made in consideration of the maximal concentration for DON in pigs as recommended in the official guidance documents published by the European Commission. Results showed that even at this low dose, DON negatively affected body weight gain and induced histomorphological alterations in the small intestines (the detailed changes induced by DON on histomorphological parameters are also addressed in **Chapter 5**, where also an intervention with non-digestible GOS is described).
The observed decrease in villus height and corresponding reduced surface area in the intestine of DON-fed piglets will impair nutrient transport and utilization, explaining a decrease in body weight gain. The negative effect of DON could also be explained by pro-emetic effects (hence DON is also denoted as vomitoxin) via neuroendocrine signalling of the nervous system [11]. There is evidence that DON might be able to reach the brain after oral administration and modify anorexigenic circuitry activity in association with anorexigenic properties of nesfatin-1 [40,41]. However, different *in vitro* and *in vivo* findings provide evidence that DON has the potency to negatively affect nutrient transporters in intestinal epithelial cells, which also may contribute to a decreased body weight gain and increased diarrhoea, induced by DON [2,42,43]. Previous studies suggested that the decreased villus height could probably be explained by villus contraction, which would aid the restoration of the impaired barrier function via reducing the surface area [37,44]. Finally, the DON-induced pro-inflammatory cytokine upregulation might also explain the poor appetite and associated reduced body weight gain after DON consumption either by affecting the central nervous system or interfering with the growth hormone axis [11]. More research is necessary to elucidate the possible mechanisms by which DON affects the piglet growth rate.

Intestinal epithelial cells are considered to be one of the primary targets following dietary DON exposure, since DON mainly enters the body via the oral route [2,45]. Indeed, qPCR analysis in our study demonstrated that the mRNA expression of different TJ proteins, inflammatory and the oxidative stress markers were affected along the piglet intestines. A low-dose DON diet in piglets induced alterations in mRNA and protein levels of TJs (**Chapter 4**). The mRNA expression patterns of different TJ proteins alongside the piglet gastrointestinal (GI) tract were enhanced by DON, except in the jejunum, in which a decreased mRNA expression of claudin4 (CLDN4), occludin (OCLN), zona occludens protein-1 (ZO-1) and ZO-2 was observed in DON-fed piglets compared to the control group. In addition to the mRNA assessments, analysis for OCLN protein expression demonstrated enhanced OCLN protein expression in the duodenum, jejunum and colon of DON-treated animals. The TJ protein OCLN is an important integral membrane protein involved in TJ stabilization and formation of a highly effective barrier [46]. Comparable studies in pigs are not available and to our knowledge we are the first to report the TJ

expression pattern in the different parts of the piglet intestine. The different effects observed in individual segments of the intestine may also be related to the kinetics of DON. DON absorption is mainly expected to occur in the upper small intestine, while the large intestines are secondarily exposed to DON via secondary routes, such as the cleavage of the DON-glucuronides and the efflux of DON from blood stream into the large intestinal lumen by ABC-transporters [10,47–49]. DON has been identified as a substrate for P-gp (ABCB1), which also suggests that absorption in the upper intestines may be regulated by P-gp, while at the same time absorbed DON reaching the systemic circulation may be actively secreted into the distal part of the intestines by the same efflux transporters [47–49].

Different *in vitro* studies confirm that the DON-induced effects on the intestinal barrier depend on the route of exposure. Diesing *et al.* [50] showed that apical and basolateral exposure of IPEC cells to DON trigger different gene response profiles, whereas Akbari *et al.* [37] observed that basolateral exposure of DON induced a more pronounced effect on intestinal permeability compared to the apical exposure in a Caco-2 cell system.

Interestingly, Waśkiewicz *et al.* [51] showed that oral administration of low DON doses to piglets resulted in its presence in gastrointestinal tissues and the distribution varied in individual tissues (duodenum, jejunum, ileum and colon) even when applied for a short period. This variation in distribution of DON along the intestines could be related to the difference in expression of P-gp transporters in the different segments of the intestines.

Regarding the molecular mechanisms that trigger the breakdown of the intestinal barrier, oxidative stress is certainly one of the general mechanisms to be addressed. The increased formation of reactive oxygen and nitric oxide can result in the degradation of membrane phospholipids, which corresponds to a decrease in membrane transport, integrity and to a hyper-permeability of the intestinal barrier [52,53]. Studies with different cell lines indicated that DON has a capacity to induce oxidative stress [54–56]. It has been demonstrated that the common target for DON (and other *Fusarium* toxins) is mitochondria [57] as one of the cellular sources for generation of reactive oxygen species (ROS) [58]. The exact mechanism for the association of DON-induced oxidative stress with the altered expressions of TJs remains to be elucidated.

It has been demonstrated that DON is capable to show immuno-modulatory effects acting as an immunosuppressive, as well as an immuno-stimulant [59]. Most *in vivo* studies have focused on a wide array of pro-inflammatory cytokines and chemokines that are detectable in different organs, like spleen, liver, kidney and lung, after high DON exposure [60–62]. The mRNA expression of different cytokines and chemokines along the intestine after dietary DON has not yet been fully investigated.

The mRNA levels of inflammatory markers, like interleukin-1 beta (IL-1β) and interleukin-10 (IL-10) were increased in duodenum of DON-fed piglets, whereas a decrease in mRNA expression was observed in jejunum. Moreover, the mRNA expression levels of cyclooxygenase-2 (COX-2) in caecum and the oxidative stress marker, heme-oxygenase1

(HMOX1), in colon were increased by DON in piglets. It has been demonstrated that this oxygenase (HMOX1) has the ability to catabolize free heme and produces carbon monoxide (CO), which gives the anti-inflammatory properties to HMOX1 by upregulation of IL-10 and IL-1 receptor antagonist (IL-1Ra) expression in mouse liver, human HepG2 cells, and mouse J774.1 macrophages [63]. Becker *et al.* [64] suggested that the decreased expression of the cytokines at low DON doses is the consequence of reduced feed consumption due to the fact that the insufficient supply of essential macro- and micronutrients could lead to the suppression of the immune response. It has been demonstrated that malnutrition inhibits the release of pro-inflammatory cytokines [65].

Mitigating the adverse effects of DON in piglets: the role of non-digestible galacto-oligosaccharides

The experimental approach to test low-dose effects of DON on the intestinal tract provides also a template for studies devoted to assess intervention strategies to combat the adverse health effects caused by DON. Considering gut health promoting effects of non-digestible GOS, which modulate the intestinal microbial population in adults, infants and healthy animals [66–68] and the protective effects of GOS on the DON-induced intestinal barrier damage [39], GOS may be a promising alternative for intervention studies against DON contamination. We investigated the effect of GOS application on the DON-induced histo-morphological alterations in the piglet intestine (**Chapter 5**). Results show that supplementation of 0.8% GOS to the DON diet fully prevented DON-induced decrease in villus area and epithelial cell area in duodenum. Furthermore, the increase in crypt depth in jejunum induced by DON could be completely prevented by addition of GOS to the diet.

The possible mechanism of GOS to diminish the adverse effects of DON in the *in vivo* model is a matter of debate. *In vitro* studies have shown that GOS accelerate TJ assembly and hence supports the integrity of the intestinal barrier [39]. In addition, our research group showed that GOS does not scavenge or bind to DON in aqueous solutions [39]. In addition to these *in vitro* experiments, in the intact animal GOS is known to be a potent prebiotic, stimulating the colonization of Bifidobacteria and Lactobacilli [68,69]. The beneficial effects of the bacterial species are most likely a result of a combination of immunomodulatory effects of the immune system, inhibition of the attachment of other pathogenic bacteria and the enhancement of barrier integrity [70].

Moreover, *in vitro* experiments with different mycotoxins suggest that probiotic bacterial species, specifically Lactobacilli, are able to sequester various mycotoxins (adherence to the bacterial cell wall) and also facilitate enzymatic degradation [71]. Considering the complex kinetic behaviour of DON with excretion of the parent compound as well as the glucuronides into the large intestines, it can be hypothesized that an improved microbiome that rapidly converts DON into its less active de-epoxidated metabolite DOM-1 may contribute to the improvement of intestinal health.

It can therefore be speculated that GOS can enhance the intestinal barrier function and

mitigate the adverse effects of DON on the intestinal architecture through microbiota-independent and microbiota-dependent mechanisms. These findings stimulated the following investigations entirely devoted to the effects of GOS on intestinal health in young piglets (as described in **Chapter 6**).

Beneficial effects of GOS on intestinal health

The effects of GOS on intestinal health and the developing immune system have been studied already in a variety of experimental models, both *in vitro* as well an *in vivo* in rodent models [72–76]. A limitation of these studies is the fact that the perinatal development of the intestines and the associated immune systems differ significantly between humans and rodents. In contrast, it is well known that neonatal piglets share many characteristics with human infants in the perinatal development of their intestinal tract [77]. Heinritz *et al.* [78] reviewed that similarity of intestinal anatomy and physiology between pig and human are related to comparable digesta transit time, analogous digestive and absorptive processes and minimum nutrient requirements (per kg of dietary DM). Furthermore, both pig and human have the same intestinal length in relation to the body weight (0.1 m/kg BW) and similar composition of the colonic microbiota.

Hence, we aimed to develop a piglet model that could demonstrate the effects of dietary GOS in the first phase of life. To this end, neonatal pigs were separated from their mothers 24 h post-partum (keeping the social contact between litter mates intact) and fed a diet supplemented with GOS at a dose that is also used in infant formulas: 0.8% [79]. Such infant formulas are used for nursing children if breast feeding cannot be provided by their mothers, due to medial indications (mastitis) or insufficient breast milk production. As it is also known that the bacterial colonization in the intestine of breastfed infants is determined within the first few days of life [80], the bifidogenic effect is considered to be one of the major beneficial effects of GOS supplemented infant formulas [81]. In the piglet study described in **Chapter 6** similar effects of early life feeding of GOS could be observed, such as the increase in the numbers of Lactobacilli and Bifidobacteria. We also observed that piglets fed a GOS diet showed a decrease in pH and an increase in short-chain fatty acid production, including butyric acid in caecum digesta. Increased acid production can lead to a lower pH, which prevent the colonization of potential pathogens [82]. Butyrate is known to show a broad range of functions, such as inhibition of inflammation, enhancement of gut barrier function and attenuation in oxidative stress through the inhibition of nuclear factor kappa B activation and histone deacetylation [83,84]. Different *in vitro* experiments indicated that low concentrations of butyrate can serve as beneficial ingredient to promote mucosal barrier function via redistribution and expression of different TJs [85,86], probably related to activation of AMP-activated protein kinase mechanism [87]. Immunomodulatory and anti-inflammatory effects of butyrate in the intestine are also described may occur via the activation of G protein-coupled receptors (GPR), which are expressed on immune cells [88,89].

In addition to the effects on the microbiota, GOS also increased the intestinal disaccharidase activity and mRNA expression of various TJ proteins along the piglet intestine. Furthermore, the intestinal defence mechanism was stimulated by GOS via an increase in beta-defensin pBD-2 in the colon, and higher sIgA levels in saliva (for details see **chapter 6**). Our findings are in line with the previously described positive effect of Lactobacilli improving gut barrier function *in vitro* via the induction of defensins [90]. Defensins, also known as antimicrobial peptides, are already expressed during pregnancy in the foetal gut, which indicates their important role in the innate immune competence during early life [91]. It has been observed that synthetic pBD-2, in a concentration-dependent manner, can inhibit the growth of pathogenic bacteria [92], which may contribute to the suppression of microbial infections. The demonstrated effect of GOS to enhance the expression of beta-defensins should be further explored as it suggests protective effects against opportunistic infections [93].
Another parameter that is influenced by the early colonization of the intestines is endogenous secretory immunolgobuline A (sIgA), which is part of the mucosal immune system [94,95]. sIgA has an important function in the establishment of intestinal homeostasis and immunity through immune protection and immune exclusion without side effects, like tissue-damaging inflammatory reactions [96]. Bifidobacteria in infants seem to be important for the synthesis of sIgA probably through the IgA producing plasmablasts [97,98]. Nakamura *et al.* [99] observed that FOS is able to increase the IgA in the mouse jejunum, ileum and colon tissue and sIgA in the luminal ileal content. sIgA binds to harmful antigens at mucosal surfaces and neutralises toxins and virulence factors [100].
Further investigations devoted to the mechanisms by which GOS can contribute to an efficient line of defence against infections through sIgA and defensins are certainly warranted. Since formula-fed infants lack the transfer of protective maternal sIgA and antibacterial peptides from breast milk [101], they would significantly benefit from dietary ingredients that support the production of endogenous sIgA and beta-defensins.

Next to the well-demonstrated effects of GOS on the expression and reassembly of the TJs, which are a prerequisite for a competent barrier function, further attention should be given to the effects of GOS on the mucus layer, secretory immunoglobulins and anti-microbial peptides. Goblet cells as the second most abundant epithelial cells are the source of mucus production providing a mechanical barrier, improving digestion by stimulating a smooth flow of nutrients along the GI tract and hosting and supporting a functional intestinal microbiome [31,96,102,103]. Previous investigation showed already that GOS application stimulated intestinal goblet cells to secrete MUC-2, trefoil factor-3, resistin-like molecule β and specific Golgi sulfotransferases [104].

Immunomodulatory effects of GOS have been acknowledged in different experiments. Vos *et al.* [72] showed immunomodulatory effects of a GOS/FOS diet through T-helper

(Th1)-dependent adaptive responses in a murine influenza vaccination model. The authors speculated that this diet may lead to better immune responses or even inhibit the excessive Th2 responses. Analysis of broncho-alveolar lavage fluid in ovalbumin sensitized rats showed that GOS effectively decreased eosinophil infiltration, which could be associated with reduced migration of activated Th2 cells [105]. Additional evidence for systemic effects of GOS were suggested in murine model experiments with house-dust mite-induced allergic asthma. Here, the oral application of GOS mitigated the induction of IL-33 (also denoted as alarmin), suppressed clinical exacerbations and supported pulmonary regulatory T cells (Tregs) function [73,74]. Vendrig *et al.* [106] demonstrated that in peripheral blood mononuclear cells (PBMCs) derived from GOS-treated foals, a lipopolysaccharide challenge resulted in lower interferon-γ and interleukin-6 mRNA expression levels compared to PBMCs of control foals. Furthermore, Lehmann *et al.* [107] showed that GOS/FOS contribute to the induction of the anti-inflammatory cytokine IL-10 and Tregs *in vitro*, which might contribute to intestinal homeostasis.

In previous [39] and current *in vitro* experiments (as described in **Chapter 3**) it is described that GOS suppress the DON-induced increase in CXCL8 secretion (marker for inflammation) in both apical and basolateral compartments of the Caco-2 cell model. The basolateral secretion of CXCL-8 is important for the recruitment of circulating neutrophils to the site of infection or damage. Moreover, apically secreted CXCL-8 is assumed to play a role in the initiation of pathways being involved in epithelial restitution prior to any impairment of barrier integrity [108–111].

All the above described investigations with non-digestible oligosaccharides provided evidence for systemic effects of oligosaccharides. Recently, our group confirmed that oligosaccharides, such as GOS, were found in serum and urine of piglets fed a milk formula enriched with GOS [112]. In addition to these *in vivo* findings, *in vitro* investigation suggested that human milk oligosaccharides as well as prebiotic oligosaccharides could be transported across the intestinal epithelial layer [113,114], which confirm the observation that human milk oligosaccharides can be detected in plasma and urine of breast-fed infants [115]. Taken together, the findings indicate new opportunities for a further evaluation of the systemic effects of oligosaccharides in various chronic disease conditions either in humans or in animals.

Mechanisms of action of GOS: evidence for microbiota-independent effects

While the microbiota-dependent effects of GOS have been studied for several years, the microbiota-independent effects of GOS become more evident. In previous experiments we demonstrated that in Caco-2 cell monolayers (and hence in the absence of microbiota) GOS are able to facilitate the reassembly of TJs after calcium deprivation and that GOS prevent the DON-induced impairment of the Caco-2 monolayer integrity [39]. These protective effects of GOS are not limited to DON, since we could also demonstrate that GOS pretreatment is able to alleviate the adverse effects of DON fungal metabolites

(3- and 15ADON) *in vitro* (for details see **Chapter 3**). In another set of experiments with Caco-2 cell exposed to heat stress, we demonstrated that GOS are able to prevent protein disruption and delocalization of E-cadherin (as a member of adherens junction proteins involved in intestinal epithelial integrity) and decreased the level of oxidative damage as indicated by the reduction of HMOX1 mRNA levels [116].
Regulation of TJs is mediated via different signalling pathways involved in the assembly, disassembly, and maintenance of TJs. A number of signalling molecules has been identified to play a role in the regulation of TJs such as protein kinase C, MAPK, AMP activated protein kinase (AMPK), myosin light chain kinase, and Rho GTPases [87,117–121]. These signalling molecules are interesting targets for future research.
Bhatia *et al.* [104] demonstrated that GOS could induce the up-regulation of trefoil factor 3 (TFF3) in human adenocarcinoma-derived cells. Intestinal TFF3 is associated with a protective effect on the intestinal barrier integrity by the modulation of the TJ proteins ZO-1, occluduin and CLDN1 and the regulation of pro-inflammatory cytokines [122].
Chapter 7 described that the addition of GOS to Caco-2 cell cultures during the phase of differentiation stimulate the expression of TJs, including CLDN1 and CLDN3, as reinforced by an earlier attainment of the TEER values that characterize the establishment of an impermeable barrier in this transwell model.
All these experiments confirm that GOS exert a direct effect on the expression of TJ proteins. In addition, GOS incubation could enhance the proliferation of intestinal epithelial cells observed by increased mRNA levels of Ki67 and cyclin D1. Functional analyses of claudins in cell cultures and in mice have suggested that claudin-based TJs may have pivotal functions in the regulation of the epithelial microenvironment (e.g. ionic conditions), which is critical for cell proliferation [123,124]. TJs can recruit signalling proteins that participate in the regulation of cell proliferation and differentiation [125]. It is believed that ZO family proteins can directly interact with proliferative processes via the regulation of cyclin D1 protein localization, protein stability and gene transcription [126].
In a calcium switch assay, we demonstrated that the GOS-induced effect on the TJ reassembly was inhibited by a MAPK ERK inhibitor. Furthermore, GOS mitigated the DON-induced phosphorylation of MAPK p38 (for details see **chapter 8**). Indicated observations provide evidence that GOS is possibly working via MAPK signalling pathways.

The mycotoxin DON can activate MAPK via a process termed the ribotoxic stress response [127]. Previously, it was demonstrated that DON is able to induce prostaglandin E_2 (PGE_2) production and COX-2 expression by elevating transcriptional activity and mRNA stability modulated by ERK and p38 signalling pathways [128]. In a review by Pestka [129], it has been discussed that ERK and p38 are both involved in DON-induced pro-inflammatory gene expression, whereas only p38 is essential for mRNA stabilization (known as the main mechanism for CXCL8 induction). We confirmed that the DON-induced CXCL8 expression and release in Caco-2 cells could be counteracted by GOS. Therefore, the

protective GOS effects on CXCL8 related to the p38 phosphorylation might be partly clarify the microbiota-independent effects of GOS.
Zenhom *et al.* [130] showed that the anti-inflammatory effects (decrease in CXCL8, IL-12 and TNF-α expression) of α3-sialyllactose or fructo-oligosaccharides in Caco-2 cells may be associated with the induction of nuclear receptor peroxisome proliferator activated receptor γ (PPRγ) and peptidoglycan recognition protein 3 (PGlyRP3).

In conclusion, the investigations presented in this thesis not only illustrate the distinct effect of DON on the intestinal barrier integrity, but also demonstrate various microbiota-dependent and microbiota-independent effects of GOS and their protective effects against exposure to the natural mycotoxin DON.

Significance of findings
Infants are considered as a high risk group for consumption of mycotoxin-contaminated food, due to their lower body mass, their higher metabolic rate, and their incompletely maturated organ functions and detoxification mechanism [131,132]. Wheat and wheat-based products, such as breakfast cereals, bread, biscuits, crackers and pasta represent the main source of DON for children in the European Union (EU) [131]. The recent investigations in Belgium devoted to the excretion of DON and its derivatives in the urine of children and adults confirmed the frequent DON exposure as all (100%) urine samples of children were positive for DON-15-glucuronide, 91% for DON-3-glucuronide, 70% for DON and interestingly in 17% of the samples even deepoxy-deoxynivalenol-glucuronide was detected [23]. Moreover, previous experiments indicated that DON is able to cross the human and animal placenta [133–137]. Feeding DON to pregnant animals (rodent studies) resulted in growth restriction and impaired foetal development [11,137]. It is also known that in addition to DON, its metabolite DOM-1 (de-epoxy DON) is also able to pass the placental barrier [138]. Intra-uterine exposure of DON is probably often followed by lactogenic exposure, as DON and DOM-1 are excreted in milk [139]. Mycotoxin excretion into breast milk has been reported for AFM_1 (the metabolite of aflatoxin B_1) and ochratoxin A (OTA) [140,141], so even co-exposure of infants to more than one mycotoxin cannot be entirely excluded.
The high risk of DON exposure in the early phase of life is of increasing concern. The finding that DON directly affects the intestinal barrier integrity and exerts an inflammatory response may have serious implications for the health status of the infant. Even long term effects cannot be excluded, as it has been shown by our group that DON is able to facilitate allergic sensitization to food proteins [142], which may contribute to enhanced allergic responses later in life in infants and adults. Reviewing the literature, it becomes evident that it is hypothesized that allergic reactions to foods can trigger or worsen the neuro-developmental disorders in paediatric patients [143].
In the light of these alarming scenarios, it seems essential to explore all possibilities to protect infants to DON exposure, and to explore means to mitigate the adverse effects of

DON. With the investigations presented in this thesis, we showed the beneficial effects of GOS not only in early phases of life addressed in the neonatal piglet model, but also the protective effects of GOS against DON-induced adverse effects both *in vivo* and *in vitro*.

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Chapter 10

General summary

General summary

Contamination of food and agricultural commodities with different mycotoxins, the secondary metabolites of various fungal species, is a worldwide increasing health concern. Deoxynivalenol (DON) is one of the most frequently occurring trichothecene mycotoxins, particularly in cereals and cereal-derived products. It is not affected by common feed and food processing methods, hence reaching unchanged the consumable products. Due to this stability and the high risk of regular exposure of adults and children, more than 40 countries have adjusted limitations and guidelines levels for DON and its metabolites in food as well as in animal feed.

DON exerts multiple symptoms, such as alterations of the intestinal, immune and nervous system. In cases of acute exposure, vomiting, anorexia, abdominal pain, diarrhea, malnutrition, headache and dizziness have been described in humans and animals. The intestinal epithelium is one of the first targets for DON and other harmful substances after oral intake of contaminated food and feedstuffs. Intestinal epithelial cells form a single layer of cells lining the gut lumen which acts as selective filter with a complex functionality. The intestinal epithelium should allow the translocation of essential dietary nutrients and beneficial substances from the lumen to the circulation. At the same time it needs to protect the internal environment and needs to constitute a tight barrier to prevent the transfer of intraluminal environmental stressors, including pathogens, antigens and different toxins. The epithelial cells are connected to each other by tight junctions (TJs), a family of proteins, which seal the space between adjacent epithelial cells. Specialized cells within the epithelial cell layer, such as goblet and Paneth cells, produce the apical protecting mucus layer and contribute to the innate defence against harmful microorganisms by the production of anti-microbial peptides. The underneath layer, the lamina propria contains the majority of immune cells of the body. It is generally assumed that any alterations and damages to the intestinal barrier by different stressors lead to inflammatory reactions, malabsorption, chronic inflammatory and immune-mediated diseases. Previous investigations have already indicated that DON is one of the stressors that can selectively affect intestinal barrier integrity.
As it seems impossible to prevent the entire mycotoxin formation on cereals and grains by plant-protection measures, various attempts have been developed to mitigate the adverse effects induced by mycotoxins. While promising results have been obtained with the use of mineral silica clays for polar mycotoxins, such as aflatoxins, the less polar DON has no efficient binding sites and hence these clay products do not significantly reduce the bioavailability of dietary DON. Alternative strategies, such as the use of pre- and probiotics were able to reduce the bioavailability of individual mycotoxins. They also mitigate other undesirable adverse effects and have gained more interest recently.

The aim of this thesis was to determine and compare DON and its naturally occurring modified forms regarding their adverse effects on Caco-2 cells, the common model for the epithelial cells lining the intestine. In addition, special attention was given to the intestinal toxicity of DON *in vivo*. One of the main objectives of this thesis, however, was also the evaluation of the efficacy of non-digestible galacto-oligosaccharides (GOS) on DON-dependent effects in Caco-2 cells, as well as in the intact animal (piglet).

In **Chapter 2**, we presented an overview of the current strategies applied to mitigate the adverse effects of DON. While this is a common practice in farm animals, particularly in poultry and pigs, albeit with varying efficacy, special attention was given to the potential application of such strategies in humans and particularly young children, as they may consume (relative to their body weight) large amounts of cereal products. Infant formulas containing GOS are already on the market, but these GOS-containing formulas may also be a promising approach for older children and adults in cases of a suspected exposure to dietary DON, especially in years with a high contamination level of the harvested grains.

In **Chapter 3**, we compared the different DON derivatives and metabolites, commonly denoted as modified forms of DON. These may be produced by fungi (like the acetylated DON forms), by plants (such as the glycosylated forms of DON), as well as by the intestinal microbiota (like DOM-1), the latter has the capacity to hydrolyze the epoxide moiety of DON. The intestinal barrier will be exposed to all these modified forms, and hence we conducted a comparative assessment of these related DON forms regarding their effect on barrier function and the inflammatory response in the well-established Caco-2 cell model. Results show that DON-glucosides as well as DOM-1 exert no major biological activity on intestinal epithelial cells over a wide concentration range. In contrast, the acetylated forms of DON are biologically active to an extend that is comparable or even may exceed, in the case of 15-acetyl-DON, the toxic effects of the parent DON molecule. The fungal metabolite 3-acetyl-DON is slightly less potent to induce adverse effects on barrier integrity. Hence, a toxic equivalence factor >1 might be appropriate for 15-acetyl-DON, whereas the equivalent factor for 3-acetyl-DON would be slightly < 1. In these experiments we also could demonstrate that GOS effectively mitigated the adverse effects of the acetylated DON-forms, which confirmed and extended previous observations of our group regarding the protective effects of GOS on the intestinal barrier and inflammation.

In **Chapter 4** we describe the outcome of an *in vivo* experiment with DON in piglets, using a DON concentration (0.9 mg/kg feed) that has been generally recognized as a safe concentration. In different parts of the intestine, clear effects of DON were however visible, both in the histo-morphometric analysis as well as in the evaluation of various functional gut health parameters. For example, Western blot analysis showed

a significant increase of occludin (OCLN) protein expression in duodenum, jejunum and colon of DON-treated animals compared to control animals, whereas the mRNA expression levels of several TJ proteins were affected along the intestine. Different inflammatory markers were investigated by qRT-PCR and the results indicated that mRNA expression of interleukin-1 beta (IL-1β) and interleukin-10 (IL-10) in duodenum of DON-fed piglets were increased. Enhanced cyclooxygenase-2 (COX-2) mRNA levels were found in caecum of DON-treated animals compared to control piglets. Among the evaluated oxidative stress markers only the mRNA expression of heme-oxygenase1 (HMOX1) was affected by DON supplementation in diet.
The results confirmed the hypothesis that the intestines are one of the major target organs for DON, and that intervention strategies for common low-dose exposure to DON in daily life, should focus on the protection of the intestinal barrier and the prevention of inflammatory reactions.

In **Chapter 5** we confirmed such a protective effect of GOS in a comparable trial with young weaned pigs. Supplementation of 0.8% GOS to the DON diet prevented the DON-induced decrease in villus area and epithelial cell area in the duodenum, whereas in jejunum, the increase in crypt depth induced by DON could be completely prevented by GOS.

Chapter 6 was entirely devoted to the characterization of beneficial effects of GOS in neonatal piglets. The model was chosen in consideration of the close resemblance of the intestinal anatomy and the perinatal early colonization and functional development of the intestines between piglets and human neonates and infants. We demonstrated that dietary GOS in neonatal piglets decreased the pH and increased butyric acid in caecum digesta and altered the intestinal microbiota by increasing the numbers of Lactobacilli and Bifidobacteria. GOS in the diet also affected the intestinal defence mechanism observed by an increased mRNA expression of porcine β-defensin-2 (pBD-2) in the colon and secretory IgA (sIgA) levels in saliva. Furthermore, histomorphological changes in the intestine of piglets induced by GOS were documented and the mRNA expression levels of various TJ proteins (claudin-1 (CLDN1), OCLN, zona occludens protein-1 (ZO-1) and ZO-2) were upregulated by dietary GOS in different parts of the intestine. From these results it could be concluded that the neonatal piglet model can be considered as a reliable model for assessing the potential beneficial effects of dietary components added to infant formulas. These dietary GOS, which are prominent components of milk, seem to balance the developing intestinal microbiota, and improve the intestinal architecture and barrier integrity.
These effects of GOS are of course also relevant for animal husbandry, as an optimal start (without the need of antibiotic intervention) is considered as one of the major parameters to guarantee optimal growth and health in later stages of life.

The direct *in vitro* effects of GOS on the intestinal epithelial layer focused on short-term exposures as described in Chapter 3 did not investigate the role of GOS during the epithelial cell maturation and differentiation phase in pre-confluent epithelial cells. Therefore, in **Chapter 7**, the effect of GOS on intestinal epithelial integrity and epithelial cell proliferation was studied in non-differentiated Caco-2 cells and followed until the stage of fully differentiated cells (long-term exposure, 17 days), resembling the development of the intestinal barrier. The epithelial integrity was increased in the GOS-treated cells as measured by an increased TEER and enhanced mRNA expression levels of the TJ proteins, CLDN1 and CLDN3. GOS also increased the epithelial proliferation, evidenced by an upregulation of Ki67 and cyclin D1 mRNA expression and a slight increase in Ki67- and cyclin D1-positive cells. In conclusion, GOS induced microbiota-independent effects on intestinal epithelial integrity and epithelial proliferation contributing to the development of the intestinal barrier in early phases of life.

In consideration of these *in vivo* and *in vitro* results demonstrating the beneficial effects of GOS on the DON-induced impairment of the intestinal barrier, we continued to speculate on the mechanisms involved in these effects. This is addressed in the final experimental chapter (**Chapter 8**), in which experiments addressing the mitogen-activated protein kinases (MAPKs) signaling pathways were targeted to unravel the possible microbiota-independent mechanism of GOS.
The underlying working hypothesis and the broader discussion of the current hypotheses on the mechanisms of action are extensively discussed in the overall discussion (**Chapter 9**).

Main findings

- DON is a very frequently occurring food and feed contaminant that target intestinal integrity resulting in the loss of barrier function and inflammation already at low (generally considered as safe) concentrations in feed (Chapter 4).

- Humans and animals may be exposed to DON and its modified forms produced by fungi, plants and the intestinal microbiota. The plant-derived DON glucosides and the bacterial de-epoxidation product DOM-1 have no significant biological activity, whereas the fungal acetylated metabolites 3- and 15-acetyl-DON almost entirely resemble the DON-mediated effects, with 15-acetyl-DON being probable even more potent due to its higher lipophilicity, whereas 3-acetyl-DON is slightly less potent to induce adverse on intestinal integrity (Chapter 3).

- In *in vitro* experiments, application of GOS can prevent the epithelial barrier impairment and corresponding inflammatory response induced by DON and its biologically active acetylated forms (Chapter 3)

- Piglets are considered as most sensitive animal species to DON. This assumption was confirmed in the feeding trials, where effects of dietary DON could be identified at levels that are generally considered as safe. Application of GOS (in a second trial) mitigated to a large extent the DON-induced histomorphological alterations in the piglet small intestine (Chapter 4 and 5).

- Piglets are a very valuable model to study the *in vivo* effects of GOS. Their perinatal development resembles that of human infants, and the physiological and morphological changes in the intestine induced by dietary GOS as measured in this presented study (Chapter 6) are in line with previous findings in human infants.

- The former working hypothesis that GOS exerts next to its well-known microbiota-dependent effects, also direct (microbiota-independent) effects was confirmed (Chapter 3 and 7). Besides the protective effects on differentiated Caco-2 cells, GOS can also modulate the epithelial cell maturation and differentiation phase in pre-confluent epithelial cells (Chapter 7). The possible molecular mechanisms, including MAPK pathways, of these effects were addressed as well, but require further investigations (Chapter 8).

- Taken together, the results presented in the current thesis allow the conclusion that non-digestible oligosaccharides (GOS) are able to mitigate many of the adverse effects exerted by the mycotoxin DON in the intestines.

Appendices

Nederlandse samenvatting

Acknowledgements

Curriculum Vitae

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Nederlandse samenvatting

Humane en dierlijke voeding is vaak met verschillende mycotoxinen (toxische stoffen geproduceerd door schimmels) verontreinigd en de mate van contaminatie neemt wereldwijd toe. Een van de meest voorkomende mycotoxinen is deoxynivalenol (DON), geproduceerd door de *Fusaria* schimmels (veldschimmels). Chemisch behoort DON tot de groep van trichothecenen en wordt niet geïnactiveerd tijdens de bereiding van voedsel (verhitten) of tijdens de bewerking van voer. DON wordt daarom niet alleen in grondstoffen (graan), maar ook regelmatig in graanproducten aangetroffen.
Om de regelmatige blootstelling van kinderen en volwassen aan DON te beperken, hebben meer dan 40 landen richtlijnen opgesteld met betrekking tot de maximale hoeveelheden van DON en zijn derivaten, zoals 3-acetyl-DON, 15-acetyl-DON, in humane en dierlijke voeding.

Blootstelling aan DON kan leiden tot veranderingen in de darmen, beïnvloeding van het immuunsysteem en het zenuwstelsel. Bij een acute intoxicatie veroorzaakt DON braken, buikpijn, hoofdpijn, diarree en duizeligheid bij mens en dier.
Het darmepitheel is vaak het eerste doelwit voor DON en andere schadelijke stoffen die met de voeding worden opgenomen. Het darmepitheel bestaat uit een laag van cellen, die door de zogenaamde *tight junctions* met elkaar verbonden zijn. Deze epitheelcellen beschikken over verschillende transportmechanismen, die de opname van voedingsstoffen mogelijk maken, maar bij een gezond individu (dier of mens) een echte barrière vormen tegen schadelijke ziekteverwekkers, toxinen en allergenen uit de voeding. Gespecialiseerde cellen in deze epitheellaag, zoals Goblet (slijmbeker) cellen en Paneth cellen, produceren daarnaast een beschermende mucus(slijm)laag met antimicrobiële eiwitten, die eveneens bijdragen aan de verdediging tegen schadelijke micro-organismen. Onder het epitheel van het darmlumen ligt de lamina propria, die de meerderheid van de immuuncellen in het lichaam bevat.

Indien de darmbarrière door toxinen verstoord wordt, zal dit gepaard gaan met ontstekingsreacties, malabsorptie en chronische ontstekings- en immuun-gemedieerde ziekten. Voorgaand onderzoek heeft reeds aangetoond dat DON de integriteit van de darmbarrière kan verstoren.
Helaas lijkt het onmogelijk om mycotoxinevorming in graan en graanproducten te voorkomen door gewas-beschermende maatregelen. Daarom zijn er verschillende strategieën ontwikkeld om de nadelige effecten van mycotoxinen te verminderen. Veelbelovende resultaten zijn behaald met mineraal-klei-producten die polaire mycotoxinen, zoals aflatoxinen, binden. Helaas blijken deze kleiproducten de biologische beschikbaarheid van DON (een apolair molecuul, dat geen bindingsplaatsen heeft) niet te verminderen. Alternatieve strategieën, zoals het gebruik van pre- en probiotica, krijgen daarom steeds meer de aandacht in dit veld, en het lijkt er op dat zij zowel de biologische beschikbaarheid van verschillende mycotoxinen kunnen reduceren, alsmede de schadelijke effecten veroorzaakt door mycotoxinen kunnen verminderen.

Het eerste doel van dit proefschrift was de verdere karakterisering en vergelijking van de schadelijke effecten van DON en zijn natuurlijk voorkomende, gemodificeerde vormen in een veelgebruikt *in vitro* model met darmepitheel (Caco-2) cellen. Daarna werd er aandacht besteed aan het effect van DON op de darmgezondheid *in vivo*. Hiervoor is een varkensmodel gebruikt. Een van de meest belangrijke doelstellingen was echter om het effect van niet-verteerbare galacto-oligosachariden (GOS) te onderzoeken zowel in de *in vitro* als ook de *in vivo* modellen, om te evalueren of deze prebiotica de schadelijke effecten van DON op het darmepitheel kunnen tegengaan.

In **hoofdstuk 2**, wordt een overzicht gepresenteerd van de huidige strategieën die toegepast worden om de schadelijke effecten van mycotoxinen (waaronder DON) te verminderen. Hoewel vele van de hier beschreven middelen (toxine-binders en enzymen) reeds gebruikt worden in de veehouderij, voornamelijk in kippen en varkens, zijn slechts enkele producten ook bij mensen getest, met name om de schadelijke effecten van aflatoxinen te verminderen. Gezien de regelmatige blootstelling van kinderen en volwassen aan de mycotoxine DON, die in graan en graanproducten voorkomt, en de negatieve invloed van DON op de darmgezondheid, werd de hypothese opgesteld, dat toevoeging van GOS aan het dagelijkse dieet een veelbelovende strategie zou kunnen zijn om de schadelijk effecten van DON te verminderen. Zuigelingenvoeding met GOS is al op de markt, maar dit zou tevens een veelbelovend product kunnen zijn voor kinderen en volwassen om de schadelijke effecten van mycotoxinen (zoals DON) in het dieet, vooral in periodes met hoge contaminatie van het geoogste graan, te verminderen.

In **hoofdstuk 3**, vergelijken we DON met de verschillende natuurlijk gemodificeerde vormen, die geproduceerd worden door schimmels (zoals 3-acetyl-DON en 15-acetyl-DON), door gewassen (zoals DON-3-glucoside), en door de darmbacteriën (zoals DOM-1). Darmepitheelcellen werden blootgesteld aan deze verschillende vormen van DON en de effecten op de integriteit van de epitheelbarrière en de ontstekingsreactie onderzocht. De resultaten laten zien dat DON-3-glucoside en DOM-1 geen effect hebben op de het darmepitheel in alle geteste concentraties, terwijl het effect van 3-acetyl-DON en 15-acetyl-DON op het darmepitheel te vergelijken is met DON. 15-acetyl-DON lijkt het effect van DON zelfs te overtreffen en 3-acetyl-DON lijkt iets minder potent te zijn in vergelijking met DON.
In deze experimenten werd ook aangetoond dat GOS deze schadelijk effecten op de darmbarrière veroorzaakt door 3-acetyl-DON en 15-acetyl-DON kan verminderen. Hierdoor worden de voorgaande observaties van onze groep betreffende de beschermende effecten van GOS op de darmbarrière en geassocieerde ontsteking bevestigd en uitgebreid.

In **hoofdstuk 4** beschrijven we de resultaten van een *in vivo* studie met biggen, waarbij de biggen een dieet met DON (0.9 mg/kg) hebben ontvangen. Hoewel deze DON dosis

algemeen beschouwd wordt als veilig voor varkens, zijn er toch duidelijke effecten van DON zichtbaar in verschillende delen van de darm. Zowel de morfologie van de darm als verschillende functionele darmgezondheidsparameters waren veranderd door het DON dieet. Zo waren bijvoorbeeld, de expressie van de *tight junction* eiwitten, zoals occludine, van ontstekingsmarkers, zoals interleukin-1 beta (IL-1β) en interleukin-10 (IL-10), en van de oxidatieve stress marker heme-oxygenase1 (HMOX1), verhoogd in verschillende delen van de darm van biggen, die een dieet met DON-contaminatie hadden ontvangen. Deze resultaten bevestigen dat de darm een van de belangrijkste doelwitten is voor DON, ook in een lage dosis. Uit dit onderzoek kan worden geconcludeerd dat interventiestrategieën tegen de veelvoorkomende blootstelling aan DON in de dagelijkse praktijk, zich zouden moeten concentreren op het beschermen van de darmbarrière en het voorkomen van ontstekingsreacties in de darm.

In **hoofstuk 5** bevestigen we een beschermend effect van niet-verteerbare galacto-oligosachariden (GOS), bekend als prebiotica, in een vergelijkbare studie met jonge, gespeende biggen, die een dieet met DON (0.9 mg/kg) hebben gekregen gedurende 10 dagen. De toevoeging van 0.8% GOS kon de negatieve effecten van DON op de morfologie in de darm voorkomen. Dit bleek uit de meting van de villilengte en diepte van de crypten, die door DON veranderden, maar na toevoeging van GOS weer normaliseerden.

Hoofdstuk 6 was totaal toegewijd aan het karakteriseren van de positieve effecten van GOS op de darmgezondheid van neonatale biggen. Dit model met neonatale biggen werd gekozen, omdat de anatomie, de vroege kolonisatie en de ontwikkeling van de biggendarm sterk overeenkomen met de ontwikkelingsstappen in de darm van baby's. In dit hoofdstuk hebben we laten zien dat GOS in het dieet van neonatale biggen een verlaagde pH en een verhoogde hoeveelheid van korteketen vetzuren in het cecum met zich meebrengt. Ook de aantallen van Lactobaccillen en Bifidobacteriën zijn verhoogd in de microbiota van de darm van biggen gevoerd met een GOS dieet. Het afweersysteem van de darm (de antimicrobiële peptiden, zoals beta-defensines, antilichamen in mucosale secreties, zoals IgA) is ook veranderd in deze behandelde biggen. Tevens zijn er verbeteringen in de morfologie van de darm te zien na toevoeging van GOS in het dieet en is de expressie van verschillende *tight junction* eitwitten (claudine-1, occludine, ZO-1 and ZO-2) verhoogd in darm ten gevolge van een GOS dieet. We kunnen concluderen dat dit model met neonatale biggen een betrouwbaar model is voor het bepalen van potentiele beschermende effecten van nutritionele componenten, die toegevoegd worden aan zuigelingenvoeding. GOS, bekend als een product met niet-verteerbare oligosachariden, die de oligosachariden in moedermelk nabootsen, lijkt de ontwikkelende microbiota in de darm te stabiliseren, de darmbarrière en darmmorfologie te verbeteren en het afweersysteem in de darm te versterken.

Ondanks dat deze proef als modelproef voor de humane gezondheidszorg werd opgezet,

zijn de effecten van GOS vanzelfsprekend ook relevant voor de veehouderij, waar GOS kan bijdragen aan een optimale start (zonder antibiotica interventie), en zo ook optimale groei en gezondheid in latere stadia zal waarborgen.

De directe *in vitro* effecten van GOS op de darmepitheellaag concentreren zich op korte termijn effecten zoals beschreven in hoofdstuk 3, waarbij geen aandacht wordt besteed aan de effecten van GOS op proliferatie en differentiatie van de darmepitheelcellen. Daarom werd in **hoofdstuk 7** het effect van GOS onderzocht op de proliferatie van de darmepitheelcellen (Caco-2 cellen) en de differentiatie naar een functionele barrière. Hierbij werd GOS gedurende de gehele differentiatie-periode (17 dagen) aan het celkweekmedium toegevoegd. De integriteit van het darmepitheel was verhoogd in de cellen behandeld met GOS, want zowel de elektrische weerstand (transepithelial electrical resistance, TEER) van de epitheellaag als de expressie van de *tight junction* proteins, claudine 1 en 3, waren verhoogd. Tevens was de proliferatiesnelheid van de darmepitheelcellen verhoogd, dit was te zien aan de verhoging in de expressie van de proliferatiemarkers Ki67 en cycline D1. We kunnen uit de bevindingen concluderen dat GOS microbiota-onafhankelijke effecten op de integriteit en de proliferatiesnelheid van het darmepitheel teweegbrengt en zodoende een bijdrage kan leveren aan de ontwikkeling van een functionele darmbarrière in de vroege levensfases.

Naar aanleiding van deze *in vitro* en *in vivo* resultaten, die beschermende effecten van GOS op de door DON verstoorde darmbarrière laten zien, hebben we geprobeerd verder te gaan in het ontrafelen van de onderliggende mechanismen. Dit heeft geresulteerd in een van de laatste hoofdstukken (hoofdstuk 8), waarin *in vitro* experimenten staan beschrijven gericht op de rol van *mitogen-activated protein kinases* (MAPKs), een signaal transductieroute, die mogelijk een rol spelen in de microbiota-onafhankelijke effecten van GOS.

Een uitgebreide discussie van de resultaten en het mogelijke werkingsmechanisme van de directe effecten van GOS op het darmepitheel wordt in hoofdstuk 9 gepresenteerd.

De belangrijkste bevindingen van dit proefschrift:

- De mycotoxine DON is een veelvoorkomende contaminant in humane en diervoeding, die de darmbarrière verstoort. Dit kan leiden tot nadelige effecten op de darmfunctie en het immuunsysteem.
- Metabolieten van DON, zoals DON-3-glucoside (gevormd in planten) of DOM-1 (gevormd door darmbacteriën) hebben geen significant effect op de darmbarrière, terwijl de aan DON-gerelateerde schimmelmetabolieten 3-acetyl-DON en 15-acetyl-DON eenzelfde of zelfs een licht geprononceerde werking hebben (hoofdstuk 3).

- In *in vitro* experimenten kan toevoeging van GOS aan het celkweekmedium de verstoorde darmepitheelbarrière en bijbehorende immuunreactie veroorzaakt door DON en zijn geacetyleerde vormen voorkomen (hoofdstuk 3).
- DON kan al nadelige effecten in de darm teweegbrengen van jonge biggen na blootstelling aan lage concentraties in de voeding. Deze lage concentraties werden voorheen als veilig beschouwd voor varkens (hoofdstuk 4).
- Toevoeging van GOS aan een DON dieet van jonge varkens laat zien dat de negatieve effecten op de darmmorfologie veroorzaakt door DON verminderd kunnen worden (hoofdstuk 5).
- De ontwikkeling van de darm in de 1e levensfase van biggen is vergelijkbaar met de darmontwikkeling bij pasgeboren baby's. Dit kon in een modelstudie met neonatale biggen bevestigd worden, waarbij de veranderingen in de biggendarm teweeggebracht door GOS-gecomplementeerd voer overeen komen met de resultaten van voorgaande studies in de mens (hoofdstuk 6). Deze studie bevestigt dat jonge biggen een zeer betrouwbaar model zijn om de *in vivo* effecten van GOS op de darmgezondheid en het immuunsysteem nader te onderzoeken.
- De werkingshypothese, dat GOS naast zijn bekende effecten op de microbiota, ook microbiota-onafhankelijk effecten vertoond, is bevestigd in de beschreven *in vitro* experimenten in hoofdstuk 3 en 7. Naast de beschermende effecten van GOS op gedifferentieerde darmepitheel (Caco-2) cellen, kan GOS ook de epitheelcelproliferatie en differentiatie beïnvloeden in pre-confluente (Caco-2) darmepitheelcellen (hoofdstuk 7).
- Voor de verdere ontrafeling van de verschillende moleculaire mechanismen, die een rol kunnen spelen bij de microbiota-onafhankelijk effecten van GOS, zoals bijvoorbeeld de mitogen-activated protein kinases (MAPKs) (hoofdstuk 8), zijn aanvullende experimenten noodzakelijk.
- Een beschouwing van alle resultaten gepresenteerd in dit proefschrift laat de conclusie toe, dat GOS vele van de negatieve effecten, die door mycotoxine DON in de darm geïnduceerd worden, kan voorkomen.

Acknowledgements

PhD sounds like a simple word, but it means a lot after four years of working hard and spending days and nights in the lab. Today is the day for writing words of thanks as the finishing touch on my thesis. The completion of this thesis would not have been possible without the generous support of many people during my PhD career.

First of all, I would like to express my special appreciation and thanks to my dear promoter, **Prof. Johanna Fink-Gremmels**. Dear Johanna, I would like to thank you for giving me the opportunity to develop my scientific character as PhD student, which made my dreams come true. Thank you for all your insightful and valuable comments and guidance throughout my PhD project which helped tremendously to shape this thesis.

My dear promoter, **Prof. Johan Garssen**. How can I appreciate all your continuous support, kindnesses, positive feedbacks and helpful comments. Dear Johan, your positive and optimistic character was always inspiring for me. Our meetings remind me of positive feelings and helpful comments. Thanks for all your help and supports during my PhD.

I would like to thank my co-promoter **Dr. Saskia Braber** for all her guidance, encouragements and constructive comments. Dear Saskia, I will remember always our conversations during the most difficult periods of my PhD career. You always helped me to overcome the challenges and be positive, strong and independent. The accomplishment of this thesis would not have been possible without your valuable support, Thank you again.

I have a special acknowledge for my third promoter **Prof. Aletta Kraneveld**, who was very supportive and helpful throughout my PhD project. Dear Aletta, thank you for being such a nice person, who always willing to help and share her knowledge in times of need.

My sincere appreciations go to my UIPS colleagues: Gert, Frank, Linette, Kim, Paula, Caroline, Laura, Atanaska, Astrid, Gerard, Mojtaba, Betty, Pim, Gemma, Mara, Marlotte, Suzan, Kristen, Suzanne, Jelle. Thank you all for making the group such a nice place to work. It was a pleasure to share with you many moments during my PhD track.

Dear Paul, as a coordinator of my PhD program you always will stay in my memory as a kind and helpful person. I do appreciate your assistance in answering my questions regarding my PhD program.

Bart, whenever I had a kind of difficulty or questions in the lab you was there to help me with your golden comments. Thanks for sharing your knowledge and experience with me.

A special acknowledgement also for our helpful secretaries at the division of pharmacology. Dear Lidija and Marga, many, many thanks to you for all your help and kindness during these years.

I also would like to extend my thankfulness to my IRAS colleagues and friends: Aneliya, Jan, Louska, Regiane, Xueqing, Amos, Dax, Cyrina, Sefanne, Rosa, Aida, Martin, Remco, Nynke, Laura W, Floris, Laura K, Manon, Rob, Fiona, Jort, Hester P, Karin, Maarke, Marieke, Martje, Sandra, Evelyn, Veerle, Adrianne, Stephan, Theo, Steven, Joris, Annick, Anke, Anne and Ingrid. Thank you all, as you all made the last 4 years an incredible and memorable period in my life.

My sincere appreciation goes also to Marjolein, Lilian and Felice for their unconditional support, help and kindness wherever and whenever needed. Thank you for everything.

Also big thanks to Raymond and Majorie for our enjoyable and friendly talks. Your friendly characters were always inspiring for me.

My dear friends Giulio and Niels, I will remember always the moments that we shared and the time we spent together within these years. Giulio your enthusiasm in science is incredible, I am sure that you will have a wonderful thesis at the end. It was so much fun going out in the weekends and evenings, spending memorable time together. Niels, you are one of the kindest friends I have ever met in my life. I am sure that our friendship is at a new starting point when I am writing these sentences. Thank you for everything and I just can wish you all the best for your future career.

I am also thankful to the colleagues from CCC project. I did appreciate our useful and constructive meetings and your input into my research project. Precilla, Paul, Margriet, Dianne, Laurien, Henk, Elisabetta and Rianne, thank you for your kind cooperation.

I would like to express my appreciation to my paranymphs, Giulio and Marlotte for their kindness, generosity and support.

I am deeply grateful to Prof. Hassan Malekinejad who supported me by all means to start this PhD, which was the biggest step in my life. We have had many good moments together at different conferences and friendly dinners (8 o'clock ☺). You were the person who introduced me to the life of science and research. Thank you for believing in me.

Lucas, you were one of my first friends in The Netherlands. Our first trip to Germany was one of the most memorable trips ever. I enjoyed a lot being together with you for a couple of months at the university campus in our small apartment. Thank you my friend.

Dear Farshad and Mazda, it was my luck to have you guys as my friends in Utrecht. We experienced unforgettable moments during our BBQ, Iranian dinners, celebrations (Shab e Yalda) and our euro trips. My great appreciation to you guys for being true and loyal friends and I am sure that our friendship will last forever.

Within the years, I have been living in The Netherlands, I had the opportunity to meet a few Iranian families. Sara and Ardalan, Maryam and Mojtaba, Farokh and Nilgun. I never forget your warm hospitality and the memorable times at your place. Thank you very much.

I wanted to extend my sincere respect to the Iranian friends here in Utrecht, with whom I spent really good times besides research. Nahid, Hamed, Mehrnoosh, Ali A, Mohaddeseh, Ali S, Vida, Kamal, Sulmaz, Hamid, Fariba, Amir A, Neda S, Yaser, Shima, Neda K, Negar and Amir R. Thank you.

Cordial thanks also to Seyed Hamed, Hadi, Vahid, Hamed, Mohammad, Mortaza, Farzad, Keyvan, Hamed, Amin, Ehsan, Shahin, Shahram, Mehdi, Reza, Tohid, Roshan and Ata. Many thanks for all nice talks in the moments that I was away from home.

The meaning of my PhD goes beyond mere research. My time in The Netherlands blessed me with the opportunity to know you guys more and more. Dear Peyman, Soheil and Nafiseh, I am not sure if I can describe with simple words your sincere friendship and kindness. Together with you guys I did not feel missing home. Thank you for your unlimited kindness and care, for being always there for me, and your unconditional friendship.

I owe a heart-felt thanks to my dear daei Farshad and his lovely family (Fery, Kimia and Arman). I am deeply grateful for your kind support and our joyful moments at beautiful Paris.

دایی فرشاد توی این سال‌ها افتخار این رو داشتم که نزدیکتر باشم بهت. ماندگارترین خاطرات و لحظات رو کنارت تجربه کردم ممنون بابت مهربانی و خوبی غیر قابل وصفی که داشتی و بودنت همیشه مایه دلگرمی بود و خواهد بود.

Finally I would like to express my appreciation and love to my family. My dear parents, you have not pass single second during my life without loving me deeply. I am so grateful to you and appreciate your care, effort and support in bringing me up and making me what I am today. I love you beyond words and I do hope you will always feel that, even when I am not around to tell you so.

پدر و مادر عزیزم، فداکاری، مهربانی، گذشت، صبر و هر چه خوبی است رو در وجود شما یافتم، بی شک هر چه آموختم در مکتب عشق شما آموختم ممنونم به اندازه یک دنیا

My dear brother, Ali aziztar az janam thank you very much for the strong support and limitless kindness. My self-confidence has been built over your shoulders, especially when I decided to continue my study abroad. My dear sister-in-law, Navideh ye aziz, I do appreciate your great understanding and inspiring discussions.

My lovely sister, Elnaz e azizam, I do appreciate all your endless kindness and care, we have had long conversations during these years, when I was far from home. Thanks for listening patiently and being with me and supporting me always. I am always very grateful of my dear brother-in-low for his calm, rational and patient character. Amir jan, thank you for your great understanding and insightful advices, which are helping me always with my big decisions in my life.

Last but not least, my lovely and cute little nephews (Farbud and Faraz e azizam) and niece (Elin junam). I am sure that being far from you, were among the most difficult moments during these years. Chatting with you online were the most joyful moments for me. Your few words and cute reactions behind the webcam always made my day. Thanks azizanam for being a new member in our family and bringing lots of fun, happiness and memorable moments for all of us.

Arash,
Utrecht, 2016

Curriculum Vitae

Arash Alizadeh was born on the 6th of April 1985 in Miyandoab, Iran. After finishing high school and passing the National University Entrance exam, he proceeded to successfully obtain his DVM degree at the Urmia University (Iran) in 2011. He joined the PhD program at Utrecht University, The Netherlands in 2012. The PhD program was a joint project between Utrecht Institute for Pharmaceutical Sciences (UIPS), Division of immunopharmacology and the Institute for Risk Assessment Sciences (IRAS) under the supervision of Prof. dr. J. Garssen, Prof. dr. J. Fink-Gremmels, Dr. S. Braber and Prof. dr. A. D. Kraneveld. During his studies, he tried to gain closer understanding of the gut health related parameters when facing with challenges like mycotoxins and opportunities like non-digestible oligosaccharides. The major findings of his PhD research are presented in this thesis.

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