Oligosaccharides to Suppress Respiratory Infections

A translational approach

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PhD thesis, Utrecht University, The Netherlands

Cover	Yang Cai
Lay-out	Yang Cai
Printed by	Ridderprint | www.ridderprint.nl
ISBN	978-94-6416-770-2
DOI	https://doi.org/10.33540/276

Printing of this thesis was kindly supported by: VanDrie Group and Utrecht Institute for Pharmaceutical Sciences (UIPS).

Oligosaccharides to suppress respiratory infections

A translational approach

Het onderdrukken van luchtweginfecties met oligosachariden

Een translationele benadering

(*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. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
woensdag 6 oktober 2021 des middags te 12.15 uur

door

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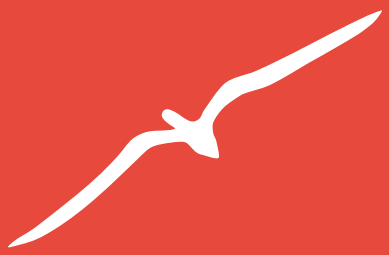
This research was partly performed in the public-private partnership 'CarboKinetics' coordinated by the Carbohydrate Competence Center (CCC, www.cccresearch.nl). CarboKinetics is financed by participating industrial partners Agrifirm Innovation Center B.V., Cooperatie AVEBE U.A., DSM Food Specialties B.V., FrieslandCampina Nederland B.V., Nutrition Sciences N.V., VanDrie Holding N.V. and Sensus B.V., and allowances of The Netherlands Organisation for Scientific Research (NWO). Research grant funding was received from the China Scholarship Council.

Contents

Dedicated to my parents, family and friends



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Chapter 1

General Introduction and Thesis Outline

1. Respiratory infections

Respiratory infections are the largest cause of childhood deaths [1, 2] and important causes of morbidity and mortality in livestock (calf) worldwide [3]. Among them, lung infection is a common and potentially life-threatening illness that considers being a major medical burden, accounting for 15% of the deaths of children younger than 5 years of age [2]. In livestock, newborn calves have more prevalent lung infections mainly due to abrupt weaning, transportation, mixing of social groups, adverse climate, and air pollution [4]. The absence of breast milk, crowded housing, and air pollution are also cofactors of lung infections in children [2].

Transmission of lung infections is thought to occur by airborne droplets/pathogens or through direct contact with colonized/infected individuals. The epithelial mucosal surface of the lungs is constantly exposed to invasive pathogens that have the potential to threaten the defense of susceptible hosts [5]. After the epithelial mucosa is invaded by pathogens, the inflammatory response occurs subsequently to recruit additional defenses. However, when these pathogens have the capacity to overwhelm the host defense, invasion of pathogens results in infections [4-6].

Important respiratory pathogens/bacteria

Mannheimia haemolytica

M. haemolytica, a Gram-negative bacterium of the *Pasteurellaceae* family, is considered the predominant cause of pneumonia in livestock (calf) [7]. *M. haemolytica* is an opportunistic pathogen and a normal inhabitant of the nasopharynx and tonsils of the host [8]. Although the mechanisms are not completely understood, predisposing factors, such as stress and an imbalanced immune system, resulting in increased multiplication and colonization of *M. haemolytica* in the upper respiratory tract. With the contribution of the possessed virulence factors (**Table 1**), including adhesins, lipopolysaccharides (LPS), and leukotoxins, *M. haemolytica* evades the host immune response, thereby invading into the lower respiratory tract and colonizing the lungs, developing infections [3, 8, 9].

Mycoplasma pneumoniae

M. pneumoniae (MP), classified as a member of the class Mollicutes, lacks a cell wall and has the ability to cause both upper and lower respiratory tract infections, including community-acquired pneumonia (CAP), which is associated with a

considerable burden of disease [10]. MP is one of the smallest self-replicating organisms and depends on obtaining host-derived critical nutrients to survive due to its limited metabolic capability and small genome. To obtain these nutrients, MP colonizes the respiratory tract by adhering to the epithelium through specialized terminal organelles containing adhesion proteins, such as P1 adhesin [11]. In addition to acquiring nutrients, MP uses virulence factors, including adhesins, community-acquired respiratory distress syndrome (CARDS) toxin, hydrogen peroxide (**Table 1**), to damage host cells, inducing ciliostasis, epithelial desquamation and inflammation [6, 10].

Host-bacteria interactions in bacterial infections

The well-developed respiratory defense is a dynamic interactive system against inhaled bacteria, including mucociliary clearance and proinflammatory responses of the respiratory epithelium, resident alveolar macrophages, and recruited neutrophils and lymphocytes [4, 5]. Nevertheless, cold air and viral coinfection impair the mucociliary clearance, leading to a marked reduction in ciliary beat frequency and mucus transport velocity [4]. In addition, the secretion of bacterial toxins and the effect of bacterial attachment impede the ciliary function. For example, *M. haemolytica* and MP express adhesins to achieve close interaction with host cells to resist mucociliary clearance [4, 6]. Alternatively, nonspecific adherence to host cell surfaces via capsular or other bacterial proteins might occur for *M. haemolytica* as well [3].

Toll-like receptors (TLRs), such as TLR2 and 4, recognize distinct pathogen-associated molecular patterns (PAMPs) derived from pathogens and detect damage-associated molecular patterns (DAMPs) released by stressed or damaged host cells [12]. These TLRs are expressed in airway epithelial cells and immune cells of both human and animals, including calves [4, 12]. The recognition of MP by human airway epithelial cells and the activation of macrophages are dependent on TLRs, particularly TLR2, resulting in the subsequent inflammatory response, such as cytokines IL-1 and IL-8 release [6]. In addition, TLR4, one of the well-characterized TLRs, senses LPS of bacteria and regulates pulmonary immunity to many gram-negative pathogens, including *M. haemolytica* [4, 12]. In pneumonic lungs of calves, *M. haemolytica*-specific LPS can be recognized by TLR4 on the surfaces of epithelial cells and alveolar macrophages, producing proinflammatory cytokines/chemokines, reactive nitrogen and oxygen intermediates, and other mediators. Subsequently, these proinflammatory cytokines/chemokines and mediators initiate an influx of neutrophils [3, 8]. The cytokine/chemokine production and leukocyte activation may either minimize respiratory infections and eliminate the bacterial pathogens

or, more often, exacerbate the disease through immunological hypersensitivity and worsening damage to the respiratory epithelium. More vigorous cytokine stimulation and cell-mediated response, leads to more severe lung injury [6]. Of course, bacteria have various tricks to evade the immune response of the host. Virulence factors synthesized by MP, such as hydrogen peroxide and superoxide radicals, act in concert with endogenous toxic oxygen molecules generated by host cells to induce oxidative stress in the respiratory epithelium; and these peroxides eventually induce lysis of host cells [6, 10]. In addition to causing oxidative bursts, the leukotoxin released by *M. haemolytica* has the capacity to induce cytotoxicity of host leukocytes, characterized by the formation of transmembrane pores and, ultimately, cell lysis. In addition, *M haemolytica*-specific LPS can be complexed with leukotoxin to enhance the leukotoxin-induced cytotoxicity [3, 8].

In addition to TLRs, particularly nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), such as NLR family pyrin domain containing 3 (NLRP3), cooperate with TLRs to regulate host defense in lung infections [13]. For example, the CARDS toxin of MP uniquely activates the NLRP3 inflammasome by colocalizing with ADP-ribosylating NLRP3, possibly leading to “hyperinflammation” (IL-1β release) [14]. Leukotoxin of *M. haemolytica* regulates voltage-gated channels of host cells and its cytotoxicity might be associated with a caspase 1–dependent pathway in host cells [8].

Notably, a well-developed airway epithelial barrier is critical in the prevention of bacterial adhesions and invasions. The airway epithelium forms a complex physicochemical barrier complemented by the mucociliary escalator to provide the first line of defense against inhaled pathogens [15]. However, respiratory pathogens exhibit strategies to impair airway epithelium leading to pathogen invasion and colonization. Pathogenic bacteria, such as *M. haemolytica* and MP, may disrupt the airway epithelial integrity through their cytotoxicity or with the help of virulence factors, causing increased paracellular permeability and damaged epithelial repair mechanisms [4, 6, 15, 16]. In addition, excessive inflammatory responses induced by pathogens also lead to the disrupted airway epithelial barrier [16].

Table 1. Functions of the representative virulence factors of *M. haemolytica* and *M. pneumoniae* (table adapted from literature [3, 4, 6, 8, 10, 16]).

Bacterial types	Virulence factors	Target cells	Functions
M. haemolytica [3, 4, 8, 16]	Adhesins	Neutrophils	Oxidative burst ↑
		Epithelial cells	1. Bind to sialoglycoprotein receptor ↑ 2. Mucociliary clearance ↓
	LPS	Leukocytes	1. Proinflammatory mediators ↑ 2. Complement and coagulation cascade ↑ 3. Cytolysis ↑
		Endothelial cells	1. Cytotoxicity ↑ 2. Vascular leakage ↑
		Epithelial cells	1. Proinflammatory mediators ↑ 2. Epithelial Integrity ↓ 3. iNOS ↑ 4. Oxidative stress ↑ 5. ATP ↑
	Leukotoxins	Leukocytes	1. Respiratory burst and degranulation ↑ 2. Proinflammatory mediators ↑ 3. Cytotoxicity ↑ 4. Transmembrane pore formation ↑ 5. Cell swelling, apoptosis and necrosis ↑ 6. Histamine release ↑
M. pneumoniae [6, 10]	Adhesins	Epithelial cells	1. Adherence ↑ 2. Mucociliary clearance ↓
	CARDS toxin	Epithelial cells	1. Ciliostasis ↑ 2. Vacuolization ↑ 3. Cytotoxicity ↑
		Leukocytes	Proinflammatory mediators ↑
	Hydrogen peroxide	Epithelial cells	1. Oxidative damage ↑ 2. Cytotoxicity ↑
		Erythrocytes	1. Denaturation of hemoglobin ↑ 2. Lipid peroxidation ↑ 3. Cytolysis ↑

ATP, adenosine triphosphate; CARDS, community-acquired respiratory distress syndrome; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide.

Possible treatments of bacterial infections

Antimicrobial administration is still a mainstay of both prevention and control of respiratory infections in animals and clinical treatment of bacterial pneumonia in humans. However, alarm bells of increasing antimicrobial resistance have been ringing with potential impact on animal and human health and food safety [17]. As early as 1976, the first report of multiple drug resistance in *M. haemolytica* in calves was reported [18]. Although antimicrobial agents are required to be used cautiously in livestock, for instance, antibiotic prescriptions can only be approved by veterinarians, the calf is still one of the livestock that uses the most antibiotics in the Netherlands [19]. Even with successfully antibiotic treatment, reduced breathing capacity hampers the welfare of calves in later life.

In humans, MP is inherently resistant to β-lactams, glycopeptides, and fosfomycin antimicrobials, which is mainly related to the lack of a bacterial cell wall. For many years, macrolides were the empirical treatments of choice for MP infections, particularly in children. The first reports of widespread macrolide-resistant *M. pneumoniae* (MRMP) appeared in Japan in the early 2000s, with subsequent spread through Asia and eventually to Europe and North America, suggesting the global spread of macrolide resistance [6]. Therefore, the dilemma of increasing antimicrobial resistance urgently requires new and innovative alternatives for antibiotics for the prevention and treatment of bacterial pneumonia/infections.

2. Non-digestible oligosaccharides (NDOs)

NDOs are low molecular weight carbohydrates, usually containing 3 to 10 sugar moieties. Many NDOs are not digested by humans due to the lack of enzymes required to hydrolyze the β-links formed among the monosaccharide units. The most famous physicochemical and physiological properties of NDOs are related to their ability to behave like dietary fibers and prebiotics, including the improvement of gut microbial composition, non-cariogenicity and low calorific value. Moreover, due to the decrease of intestinal pH caused by their fermentation, NDOs exhibit the ability to reduce the growth of pathogenic bacteria, increase the populations of *bifidobacteria* and *lactobacilli*, and increase the utilization of minerals in the gut. In addition, NDOs are also associated with a lower risk of (gastrointestinal, respiratory, and urogenital) infections and exert immunomodulatory properties [20, 21]. More details of the beneficial effects of NDOs are described in **Chapter 8**. Here, a short overview of the beneficial effects of specific NDOs, such as human milk oligosaccharides (HMOs), galacto-

oligosaccharides (GOS), and fructo-oligosaccharides (FOS), is given below.

HMOs, GOS, and FOS

HMOs are gifts from mother to newborns and are the first group of NDOs consumed by humans after birth. Breastfeeding infants ingest mother milk several times per day, bathing the nasopharynx and mouth for several minutes at each feeding with a solution high in HMOs, which might inhibit local adherence of pathogenic bacteria [22]. Breastfed infants have lower incidence of respiratory diseases, including respiratory infections, during early life [23-25]. In addition to the well-known immunomodulatory and prebiotic properties of HMOs [21], approximately 1-5% of HMOs is absorbed by the intestine into the systemic circulation [26], directly interacting with pathogens, immune cells, and epithelial cells outside the intestine [21].

Various strategies have been used to mimic the beneficial effects of HMOs, including GOS and FOS, so far have been supplemented in dietary products and infant formula [27, 28]. Commercial production of GOS has been achieved from lactose by the action of β-galactosidases [20]. FOS can be produced from the controlled enzymatic hydrolysis of the polysaccharide inulin, which can be extracted from chicory roots [29]. Both GOS and FOS exert many beneficial properties. For example, both can stimulate the growth of *bifidobacteria* and *lactobacilli* and support the development of the immune system. Moreover, both can inhibit the inflammatory responses and prevent epithelial barrier dysfunction in the intestine; in particular, GOS have the property of inhibiting the adhesion of pathogens to intestinal epithelial cells [21, 28]. There is no doubt that GOS/FOS mixture has similar properties, and even recently, a reduction in airway inflammation after oral administration of this mixture was demonstrated [30, 31]. There are several studies investigating the supplementation of GOS and/or FOS in human respiratory infections mainly focusing on clinical observations, including the reduced frequency of respiratory infections and antibiotic prescriptions in infants, as well as the decreased duration and symptoms of cold or flu in university students [32-34]. These impressive observations encourage GOS and/or FOS to become attractive candidates in the prevention and clinical treatment of respiratory infections. However, the effects of NDOs in some studies are inconsistent; for example, a combination of probiotics and GOS showed no preventive effect on allergic diseases in infants [35]. In a study with mice, a mixture of FOS and inulin did not affect the immune response of delayed hypersensitivity in an influenza vaccination model [36]. Therefore, more in-depth studies are required to investigate possible effects and potential mechanisms of action of these NDOs in respiratory diseases (infections).

3. Aims and Outline of the Thesis

The main aim of this thesis is to expand the current knowledge on the ability of NDOs in strengthening the respiratory defenses, focusing on the protection of the airway epithelial cells and subsequent inhibition of the inflammatory responses. The rationale of the *in vivo* study is to investigate the effects of NDOs (e.g., GOS and FOS) on calves under conditions of high respiratory infection pressure.

In line with this objective, the following investigations are presented:

Part I. *Modelling Respiratory Infections ex vivo and in vivo.*

Cellular and animal models are essential in the characterization of the pathophysiology of diseases, like human respiratory infections, and the evaluation of novel therapeutic agents. In **part I**, *ex vivo* and *in vivo* models of respiratory infections are described. In **Chapter 2**, an *ex vivo* method for isolation and culture of primary bronchial epithelial cells (PBECs) from calves is established with *M. haemolytica* and LPS as triggers to mimic the *in vivo* situation. In **Chapter 3**, immunological and inflammatory responses are investigated in a natural occurring respiratory infection model in calves.

Part II. *Pathogenesis of Respiratory Infections and Early Life Intervention.*

In **part II**, the mechanisms and effects of GOS and FOS are investigated in the *ex vivo* and *in vivo* models of respiratory infections. In **Chapter 4**, the effect of GOS on the NLRP3 inflammasome activation in calves with lung infections and *M. haemolytica*-exposed PBECs are studied. GOS exert the ability to inhibit NLRP3 inflammasome activation leading to interesting anti-inflammatory effects *in vivo* and *in vitro.* In **Chapter 5**, GOS are innovatively administered to the respiratory tract through the nose in calves with lung infections. GOS display reduction of the respiratory pathogen numbers and airway inflammation *in vivo*, as well as have the capacity to lower *M. haemolytica* viability and prevent the disrupted bronchial epithelial barrier *in vitro*. In **Chapter 6**, the elegant anti-inflammatory effect of FOS is observed in naturally exposed calves and in *M. haemolytica*-exposed PBECs, which might be related to the interference with the TLR5-mediated signaling. *In vivo* data in **Chapter 3-6** are part of a larger calf trial. In this large calf trial, several experimental groups were tested. Results of comparisons of different (dietary) interventions with the same non-intervened control group have been presented in **Chapter 3-6**.

Part III. *Relevance to Human Respiratory Infections and Health.*

In **part III**, the link with the human situation is described by using human respiratory pathogens and describing the beneficial effects of dietary fiber on human health. In **Chapter 7**, the bacteriostatic effect of FOS and the bactericidal effect of GOS on MP and macrolide-resistant MP are demonstrated, including the possibly enhanced anti-bacterial efficacy of erythromycin by GOS *in vitro*. In **Chapter 8**, a broader view on microbiota-dependent and -independent effects of dietary fiber, such as NDOs, on human health is presented. Finally, in **Chapter 9**, the most relevant findings and clinical relevance are discussed. Prospects are presented to a closer understanding of NDOs in the prevention and treatment of human and animal respiratory infections.

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Part I

Modelling Respiratory Infections ex vivo and in vivo

2

Chapter 2

Mannheimia Haemolytica and Lipopolysaccharide Induce Airway Epithelial Inflammatory Responses in an Extensively Developed *ex vivo* Calf Model

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This chapter is published in *Scientific Reports*, 2020. 10:13042.

Abstract

Respiratory infection is associated with inflammation and damage to the bronchial epithelium characterized by an increase in the release of inflammatory mediators and a decrease in airway barrier function. Our objective is to optimize a method for the isolation and culture of primary bronchial epithelial cells (PBECs) and to provide an *ex vivo* model to study mechanisms of airway epithelial inflammation. PBECs were isolated and cultured from the airways of calves in a submerged cell culture and liquid-liquid interface system. A higher yield and cell viability were obtained after stripping the epithelium from the bronchial section compared to cutting the bronchial section in smaller pieces prior to digestion. *Mannheimia haemolytica* and lipopolysaccharide (LPS) as stimulants increased inflammatory responses (IL-8, IL-6 and TNF-α release), possibly, by the activation of “TLR-mediated MAPKs and NF-κB" signaling. Furthermore, *M. haemolytica* and LPS disrupted the bronchial epithelial layer as observed by a decreased transepithelial electrical resistance and zonula occludens-1 and E-cadherin expression. An optimized isolation and culture method for calf PBECs was developed, which cooperated with animal use Replacement, Reduction and Refinement (3R’s) principle, and can also contribute to the increased knowledge and development of effective therapies for other animal and humans (childhood) respiratory diseases.

Keywords: Pulmonary infection; Inflammatory mediators; Toll-like receptors; Barrier function; Primary bronchial epithelial cells

Introduction

Respiratory infections, such as pneumonia and bronchiolitis, are complex, multifactorial disorders caused by viral and/or microbial pathogens, an impaired immune system, as well as environmental and genetic factors. It is generally accepted that inflammation and damage of bronchial epithelium contribute to the development of respiratory infections in animals and humans [1-6]. The airway epithelium plays a central role in the maintenance of airway integrity and acts as a physical barrier to protect the lungs against inhaled (infectious) particles. Bacterial and viral infections can induce the release of inflammatory cytokines and chemokines, including interleukin (IL)-8, IL-6 and tumor necrosis factor (TNF)-α from airway epithelial cells. The major cause of epithelial barrier breakdown during lung inflammation is related to the damage of tight and adherens junctions [7-10].

Due to the risk of development and the presence of antibiotic resistance, new avenues have to be explored to tackle (opportunistic) infections. The primary culture of bronchial epithelial cells is of particular importance, allowing the characterization of pathogenic infection in airway epithelium, advancing our knowledge of airway inflammation, and developing new intervention strategies.

Because of the ethical and practical concerns, especially among vulnerable population groups, including children, it is highly challenging to obtain primary tissue for isolating primary bronchial epithelial cells (PBECs) from infants and children, which hinders progress in research. However, the bovine epithelium can be obtained on a regular basis in sufficient quantities from calves slaughtered for food consumption. Studies in calves have been shown predictive for respiratory infections for decades [11, 12]. The calf model of respiratory syncytial virus (RSV) infection has been described as a relevant model for preclinical testing of vaccine candidates related to the similarities between human (h)RSV and bovine (b)RSV [12]. Related to the animal use Replacement, Reduction and Refinement (3R's) principle, PBECs from calves cultured *ex vivo* may be used as a respiratory disease model for investigating pathophysiological and immunological characteristics.

Toll-like receptors (TLRs) play a major role in bacterial recognition and epithelial innate immunity, where TLR4 primarily recognizes lipopolysaccharide (LPS) and TLR5 recognizes bacterial flagellin [13, 14]. Activation of TLRs by bacteria leads to TLR-mediated signal transduction pathways in epithelial cells (e.g., via phosphorylated mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB), and subsequent production of cytokines and chemokines that recruit and activate the innate and adaptive immune system and regulate the

barrier function of epithelial cells [9]. *Mannheimia haemolytica* is a Gram-negative bacterium associated with lung infections in neonatal calves and is responsible for economic losses in the global livestock industry [15]. *M. haemolytica* and its released virulence factors (e.g., LPS) play an important role in the pathogenesis of bovine lung infections [16]. Acute pneumonia caused by *M. haemolytica* is characterized by a decline in the innate immune function, dysfunction of airway epithelium and a large influx of inflammatory mediators into the airways [17, 18]. However, it is not well-described whether *M. haemolytica* can affect TLR signaling, impede normal epithelial barrier function and promote inflammation in an *in vitro* model with primary airway epithelial cells.

The aim of this study is to optimize a method for the isolation and culture of PBECs and to provide an *ex vivo* model to study mechanisms of airway epithelial inflammation induced by *M. heamolytica* and LPS. A detailed description of two isolation methods (stripping and cutting the bronchial section prior to digestion) of bovine PBECs was given. Thereafter, we examined the effect of *M. haemolytica* and LPS on cellular viability, the production of inflammatory mediators, barrier function and the associated mechanisms in the PBEC model. *M. hae*molytica and LPS can induce the production of inflammatory cytokines/chemokines (IL-8, IL-6 and TNF-α), and “TLR-mediated MAPKs and NF-κB" signal transduction may be one of the possible mechanisms of action. *M. haemolytica* and LPS reduced the transepithelial electrical resistance (TEER) and decreased expression of the tight junction protein, zonula occludens-1 (ZO-1) and adherens junction protein, E-cadherin.

Results

Establishment of primary cultures of calf bronchial epithelium

To better understand the respiratory infections in the calf, we first established an *ex vivo* calf bronchial epithelium infection model. Hereto, bronchial sections of a similar size and weight were cut from the primary bronchus of freshly slaughtered calves and subjected to the cell isolation procedure depicted in **Figure 1**. One approach to isolate fresh PBECs involved stripping of the epithelium from the bronchial section followed by treatment with a digestion buffer (**Figure 1A**, the strip method). Alternatively, the bronchial section was first to cut into smaller fragments and then subjected to enzymatic digestion (**Figure 1B**, the cut method). Isolation of PBECs following the stripping of the epithelium resulted in a significantly higher yield and cell viability compared to the enzymatic digestion of complete bronchial explants (**Figure 1C**, 2.2 ± 0.2 x 10^6 cells/mL vs 13.7 ± 0.6 x 10^6 cells/mL; **Figure 1D**, 75.5 ± 1.6% vs 94.1 ± 0.3%; n=15). Due to the high-efficiency characteristics, the strip method for isolating PBECs was used in all subsequent experiments.

Isolated cells (obtained via the strip method) were incubated with short-incubation medium (1h) and thereafter successfully propagated in serum-free medium (2-3 days). After 2-3 days, PBECs were cultured in FBS medium for another 2-3 days and formed clear networks of attached cells in the submerged cell culture (SCC) system. Both the initial and passaged PBECs exhibited a polygonal, cobblestone appearance, typical of epithelial cells as depicted in **Figure 2A**. The epithelial lineage identity of the PBECs was confirmed by immunofluorescence staining of the cells for the epithelial marker cytokeratin. On average, 99.3 ± 1.4% (n=6) of the PBECs stained positive for the expression of this cytokeratin (**Figure 2B**).

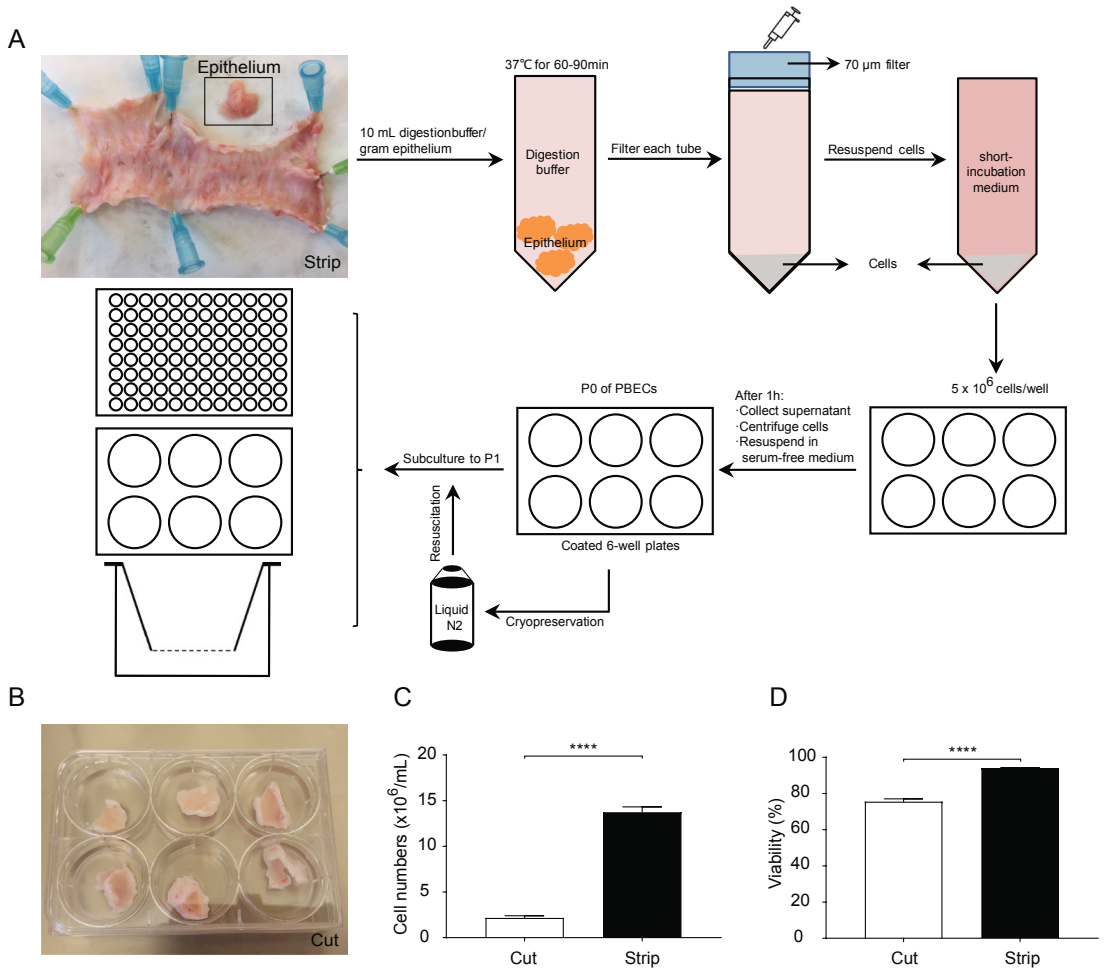


Figure 1. Establishment of primary cultures of calf bronchial epithelium. (**A**) Overview of isolation and culture of PBECs. The epithelium was first stripped from the bronchial section (**A**), or the bronchial section was cut into smaller fragments (**B**). After digestion, the total cell numbers (**C**) and cell viability (**D**) were significantly higher in the stripped bronchial epithelium compared to the bronchus that was cut into small fragments. ****P<0.0001 (unpaired Student's t-test). Data are presented as means ± SEM (n=15 donor calves).

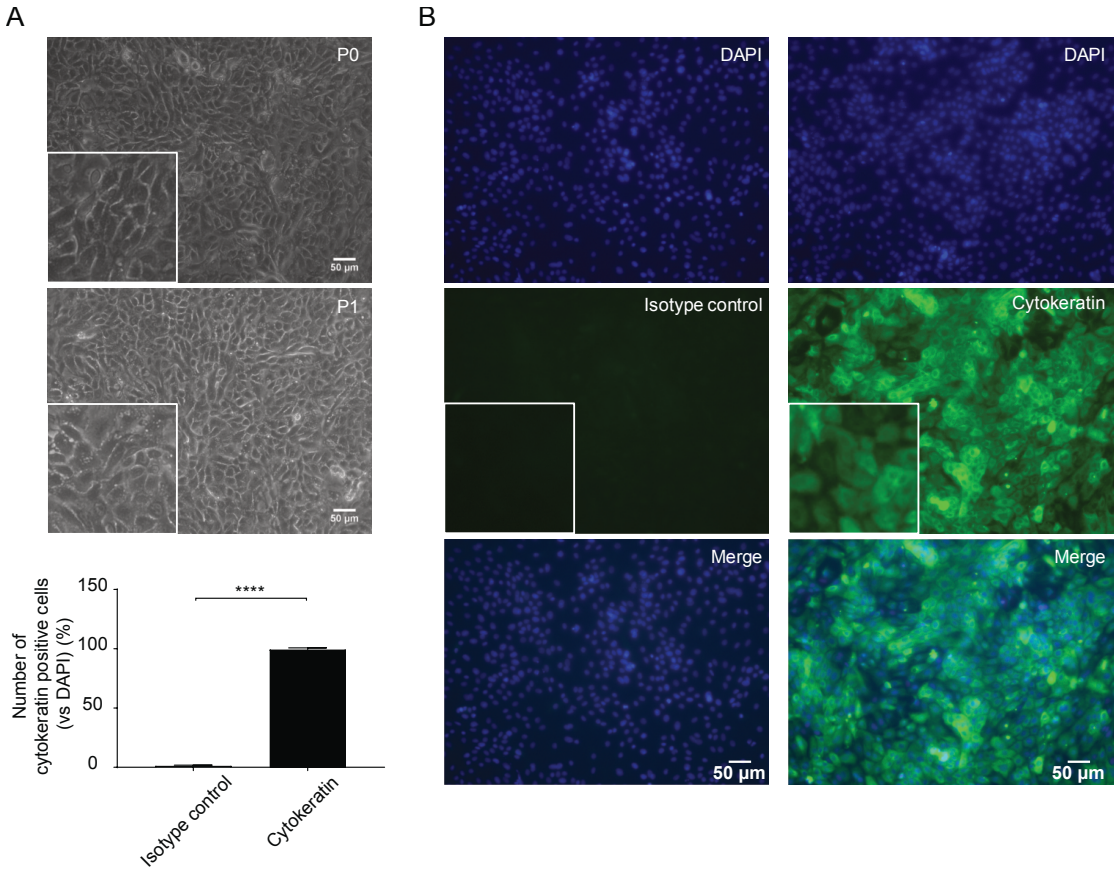


Figure 2. PBECs form a clear network with attached cytokeratin-positive cells. (**A**) Representative microscopic picture of *passage* 0 (P0) and *passage* 1 (P1) of PBECs in the SCC system. (**B**) P1 of PBECs stained by isotype control or cytokeratin antibody (green), followed by counterstain with 4', 6-diamidino-2-phenylindole (DAPI), which illuminates cell nuclear material (blue). Original magnification, 200x; higher magnification, 400x. ****P<0.0001 (unpaired Student's t-test). Data are presented as means ± SEM (n=5 donor calves).

Exposure of PBECs towards *M. haemolytica* and LPS

To investigate the potential of the cultured PBECs as an infection model, we infected the cells with increasing concentrations of the respiratory pathogen *M. haemolytica.* Air-dried cytospin preparations with *M. haemolytica*-infected PBECs showed adhesion and invasion of *M. haemolytica* to the epithelial cells (**Figure 3E**). To assess possible cell toxicity caused by *M. haemolytica*, cellular survival (MTT assay) and lactate dehydrogenase (LDH) release from the PBECs was measured after 24h of infection. At *M. haemolytica* concentrations of >10^5 CFU/mL, cellular survival dropped in a concentration-dependent fashion in PBECs obtained from four different calves (**Figure 3A**). This coincided with an

increase in the LDH release (**Figure 3B**). At concentration of LPS of >10 µg/mL, the PBECs showed a lower survival rate (**Figure 3C**) and a higher LDH release (**Figure 3D**) for PBECs obtained from three different calves, resembling the toxic effect of the bacterial pathogen on the cells. Therefore, *M. haemolytica* concentrations of 10^5 CFU/mL and 10 µg/mL LPS will be used in future experiments.

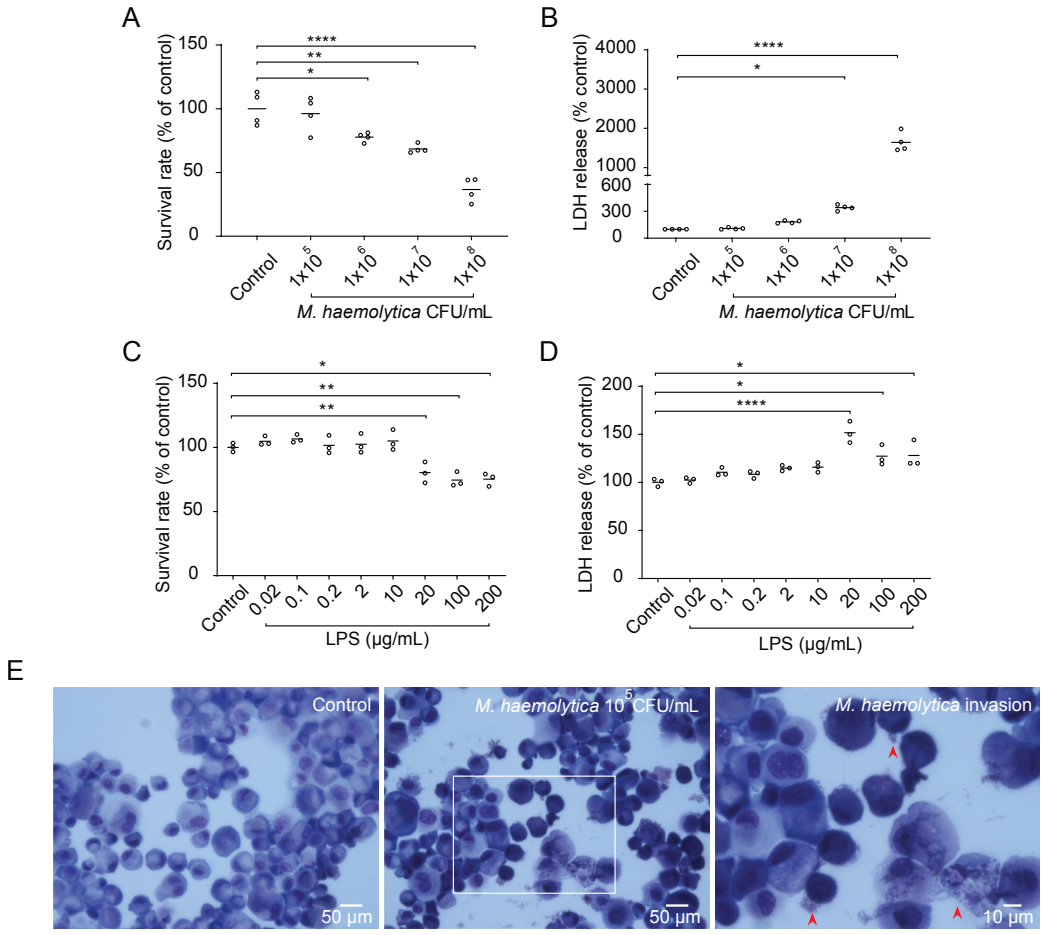


Figure 3. Exposure of PBECs towards *M. haemolytica* and LPS. *Passage* 1 (P1) of PBECs were incubated with increasing concentrations of *M. haemolytica* (1 x 10^5-10^8 CFU/mL) (**A-B**) and LPS (0.02-200 µg/mL) (**C-D**) for 24h in the SCC system and (**A and C**) survival rates were determined by the percentage of MTT levels in control and LPS/*M. haemolytica*-treated PBECs. (**B and D**) LDH levels were measured in the supernatants of control and LPS/*M. haemolytica*-treated PBECs. (**E**) Representative microscopic pictures of P1 of control and *M. haemolytica*-treated PBECs on air-dried cytospin preparations. **P*<0.05; ***P*<0.01; *****P*<0.0001 (one-way ANOVA). Data are presented as means ± SEM (n=3-4 donor calves).

Inflammatory pathways of PBECs activated by *M. haemolytica* and LPS

Infection of PBECs with *M. haemolytica* (1 x 10^5,1 x 10^6, and 1 x 10^7 CFU/mL) increased the IL-8 production by the cells in a concentration-dependent fashion (**Figure S1**). One of the factors that may contribute to IL-8 production is bacterial LPS. Addition of increasing concentrations (2-200 µg/mL) of purified LPS to the cultured PBECs indeed induced a concentration-dependent release of IL-8 in the cell culture supernatant (**Figure S2**). The optimal concentrations of *M. haemolytica* (1 x 10^5 CFU/mL) and LPS (10 µg/mL) as mentioned above did not only increase the IL-8 release (**Figure 4C**) but also enhanced the IL-6 and TNF-α production by PBECs (**Figure 4A** and **B**).

To learn more about the cellular pathways that are activated by *M. haemolytica* and LPS, we determined the expression of TLRs in the PBECs and the phosphorylation of MAPKs and NF-κB p65 during exposure of the cells to the pathogen and LPS. Western blotting of cell lysates showed that the cultured PBECs express both TLR4 and TLR5 innate immune receptors. Infection of the cells with *M. haemolytica* increased both TLR protein levels, whereas LPS stimulation only resulted in a significant increase in TLR4 protein levels (**Figure 4D**). On the other hand, both *M. haemolytica* and LPS promoted the phosphorylation of p38, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH2-terminal kinase 1/2 (JNK1/2) MAPK and NF-κB p65 (**Figure 4D**). Thus, the observed cellular release of the different proinflammatory mediators may result from a TLR-dependent activation of MAPKs and NF-κB p65 pathways in PBECs.

Moreover, stimulation of PBECs with flagellin (1, 10, 100, 1000 ng/mL), a TLR5 agonist, increased the IL-8 production but did not affect the LDH release and survival rate. In addition, flagellin (10 ng/mL) also increased the TNF-α and IL-6 production by PBECs (**Figure S3**).

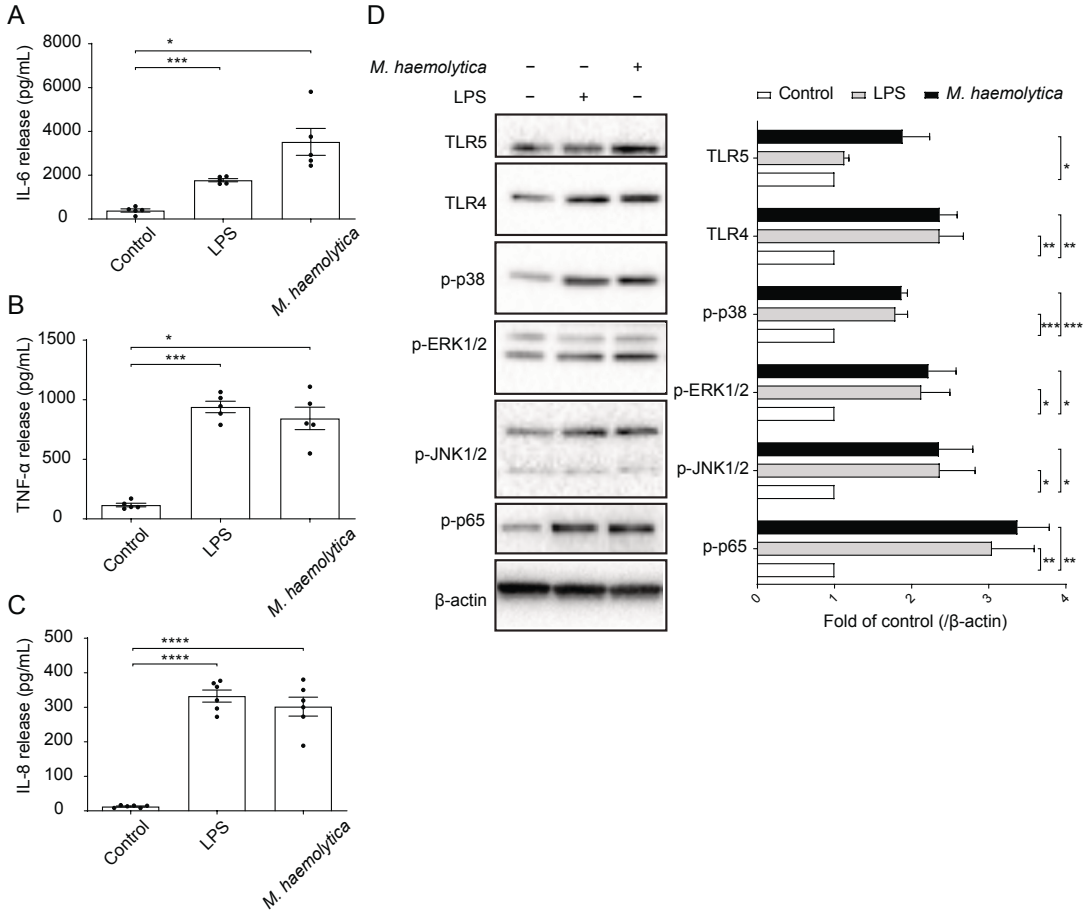
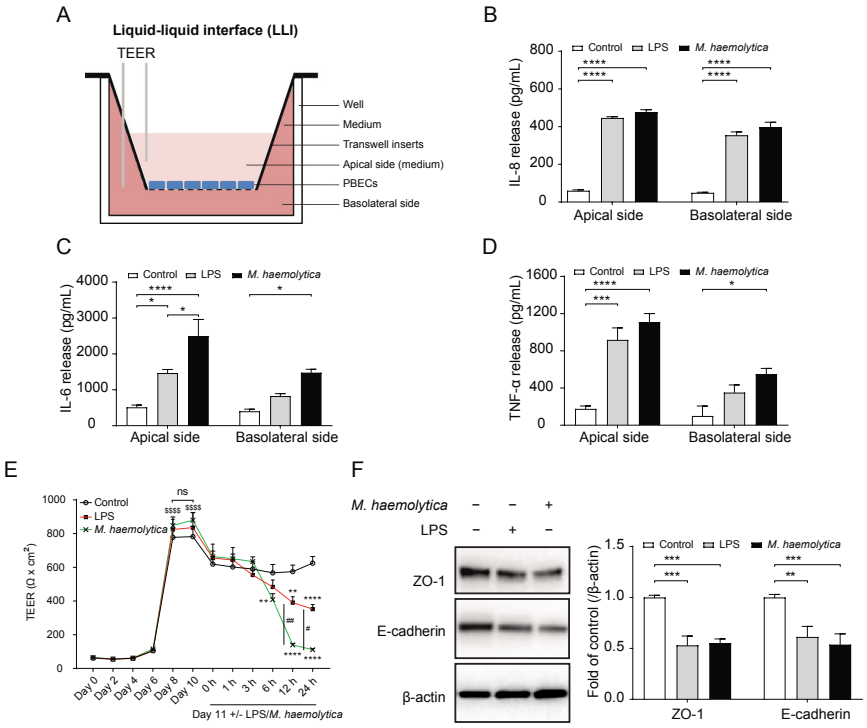


Figure 4. Inflammatory pathways of PBECs activated by *M. haemolytica* and LPS. *Passage* 1 (P1) of PBECs were incubated with LPS (10 µg/mL) and *M. haemolytica* (1 x 10^5 CFU/mL) for 24h in the SCC system and (**A**) IL-6, (**B**) TNF-α and (**C**) IL-8 levels in the supernatants of control and LPS/*M. haemolytica*-treated PBECs were assessed by ELISA. (**D**) Expression of TLR4/5 and phosphorylation of p38, ERK1/2, JNK1/2 and NF-κB p65 were determined by western blot in control and LPS/*M. haemolytica*-treated PBECs (original blots are shown in supplementary Figure S5A). **P*<0.05; ***P*<0.01; ****P*<0.001; (**A**–**C**) one-way ANOVA; and (**D**) two-way ANOVA. Data are presented as means ± SEM (n=5-6 donor calves).

Effects of *M. haemolytica* and LPS on the barrier function of PBECs

As *in vivo,* PBECs are part of a polarized monolayer of cells that form the bronchial lining, that may become disrupted during infection, we established PBEC growth on 0.4 µm semipermeable membranes in the liquid-liquid interface (LLI) system (**Figure 5A**). In this system, it is possible to determine the integrity of the epithelial barrier and to differentiate between the apical and basolateral release of cytokines/chemokines. As an indicator of epithelial barrier integrity, TEER was measured every two days of culture. After seeding the cells, TEER value started to increase from day 6 and values stabilized at around 600 $\Omega \cdot cm^2$ on day 11 (**Figure 5E**). The administration (day 11) of *M. haemolytica* (1 x 10^5 CFU/mL) or LPS (10 µg/mL) to the apical compartment significantly decreased the TEER value after 6h, 12h, 24h and 12h, 24h compared to the control PBECs, respectively (**Figure 5E**). Especially, 12h and 24h *M. haemolytica* exposure induced a dramatic decrease in TEER value compared to the control and LPS-treated PBECs (**Figure 5E**) even reaching the low TEER value after seeding. To learn more about this effect, the tight junction protein ZO-1 and the adherens junction protein E-cadherin were analyzed by Western blot and immunofluorescence staining. This showed that both *M. haemolytica* and LPS induced a significant decrease in the expression of the tight junction ZO-1 (**Figure 5F** and **G**) and adherens junction E-cadherin (**Figure 5F** and **H**).



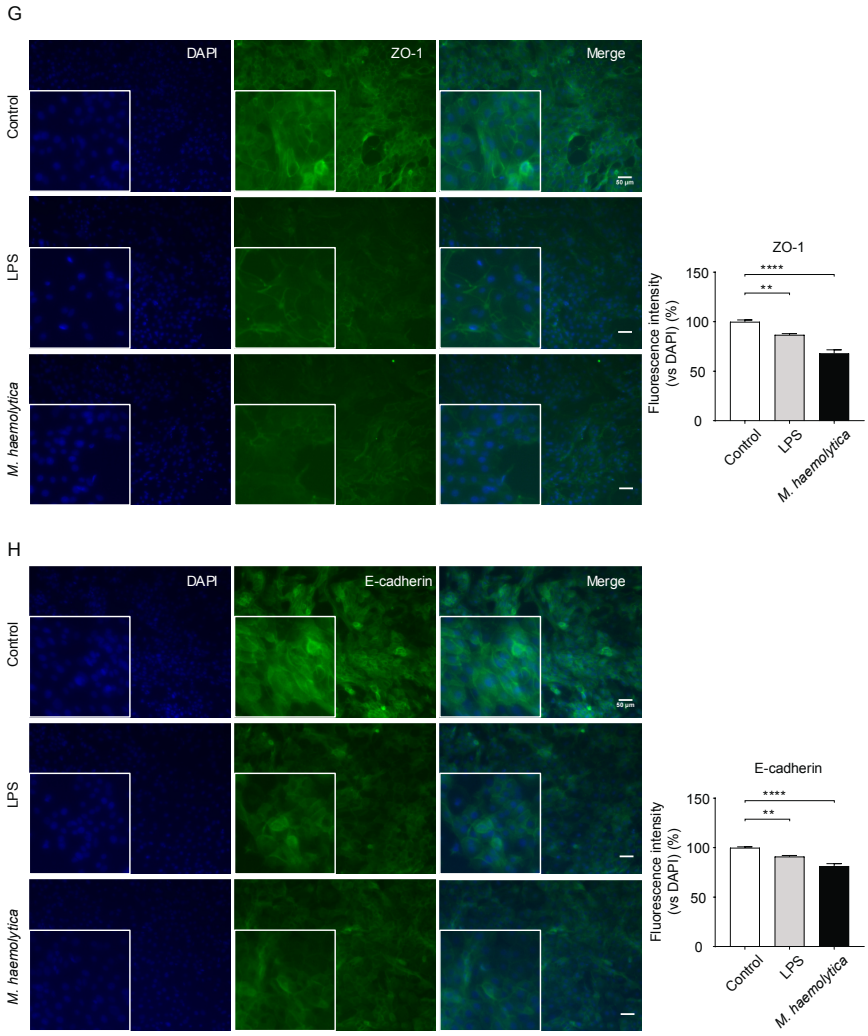


Figure 5. Effects of *M. haemolytica* and LPS on the barrier and immune function of PBECs. (**A**) P1 of PBECs were cultured on transwell inserts and FBS medium was added to both apical and basolateral compartments. (**B-D**) IL-8, IL-6 and TNF-α release in the supernatants of the apical and basolateral compartments from control and LPS/*M. haemolytica*-stimulated PBECs was measured by ELISA. (**E**) TEER value was measured every 2 days until day 10. At day 11, LPS (10 µg/mL) or *M. haemolytica* (1 x 10^5 CFU/mL) were added to the apical side for 24h and TEER value was measured at different timepoints (1h, 3h, 6h, 12h, and 24h) in control and LPS/*M. haemolytica*-treated PBECs. (**F**) Expression of ZO-1 and E-cadherin were determined by western blot in control and LPS/*M. haemolytica*-treated PBECs (original blots are depicted in supplementary Figure S5B). (**G** and **H**) P1 of PBECs were stained for ZO-1 (**G**) and E-cadherin (**H**) (green), followed by counterstaining with DAPI (blue). The fluorescence intensity was analyzed in representative pictures from PBECS obtained from six calves. Original magnification, 200x; higher magnification, 400x. $$$$*P*<0.0001 (Day 8 or 10 vs Day 6); #*P*<0.05; ##*P*<0.01 (*M. haemolytica* vs LPS group); ***P*<0.01; ****P*<0.001; *****P*<0.0001 (6, 12 or 24h vs 0h); (**B**–**F**) two-way ANOVA; and (**G-H**) one-way ANOVA. Data are presented as means ± SEM (n=4-6 donor calves).

Differential release of IL-8, IL-6 and TNF-α by PBECs

To investigate whether exposure of polarized PBECs to the pathogen or LPS resulted in a differential release of cytokines into the apical or basolateral cell compartment, *M. haemolytica* (1 x 10^5 CFU/mL) or LPS (10 µg/mL) were administered to the LLI system. After 24h, the IL-8 release by PBECs at both the apical and basolateral compartments was significantly increased after exposure to both *M. haemolytica* and LPS (**Figure 5B**). The IL-6 and TNF-α release by PBECs into the apical compartment was also significantly increased after both *M. haemolytica* and LPS stimulation. However, the release of IL-6 and TNF-α into the basolateral compartment was only significantly increased after stimulation with *M. haemolytica*, but not LPS (**Figure 5C** and **D**).

After 24h stimulation, LPS (10 µg/mL) did not affect the cellular survival (**Figure S4C**) and LDH release in both apical (**Figure S4A**) and basolateral (**Figure S4B**) compartment of the PBECs. However, *M. haemolytica* (1 x 10^5 CFU/mL) tended to decrease the cellular survival (**Figure S4C**) and increase the LDH release in the apical compartment (**Figure S4A**) but not in basolateral compartment (**Figure S4B**), however these effects were not significantly different from control values.

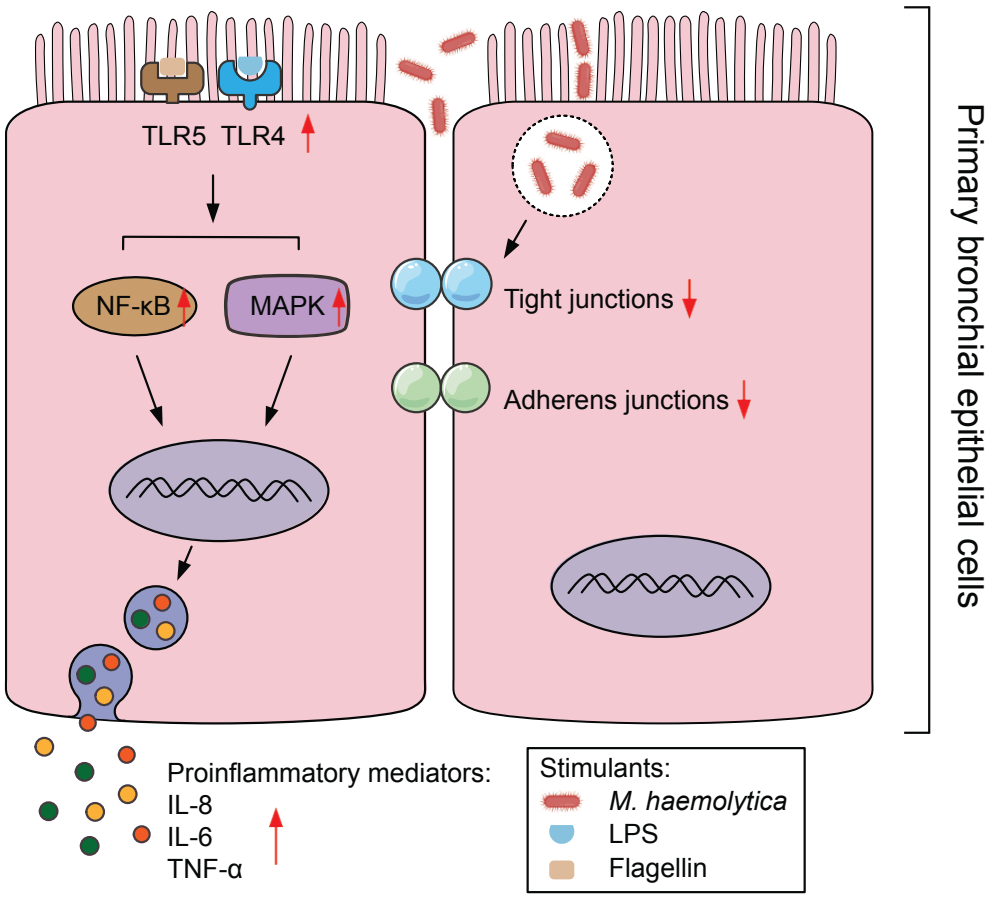


Figure 6. Schematic overview of the postulated mechanism of *M. haemolytica*-induced dysfunction of epithelial barrier and release of pro-inflammatory mediators in PBECs. *M. haemolytica* promotes the release of proinflammatory mediators (e.g., IL-8, IL-6 and TNF-α) in PBECs, which might be related to the phosphorylation of MAPK proteins and NF-κB p65 by the activation of TLR4/5. The invasion of PBECs by *M. haemolytica* and the *M. haemolytica*-induced release of proinflammatory cytokines/chemokines reduce the barrier function of epithelial cells, thereby weakening the resistance of epithelium to pathogens, and enhancing the development of inflammation in PBECs.

Discussion

In the present study, different calf primary bronchial epithelial cell models were developed and optimized, which can be used to investigate the pathogenesis and mechanisms of respiratory diseases. First, the methodology of isolation and culturing PBECs was established and thereafter *M. haemolytica* and LPS were used as stimulants to induce an *in vitro* inflammation/infection. Inflammation and damage of bronchial epithelium contribute to the development of respiratory diseases. Bacterial and viral pathogens causing these diseases can modulate the expression of TLRs and the release of inflammatory cytokines and chemokines from airway epithelial cells [5, 7-9, 19]. In this study, the inflammatory response of PBECs was tested via *M. haemolytica* and LPS exposure in two different cell culture systems. The effects of *M. haemolytica* and LPS on cellular viability, cytokine and chemokine release, barrier function and the associated mechanisms (TLRs, MAPKs, NF-κB) of the PBECs were examined (**Figure 6**).

In the current study, fibroblast contamination was one of the largest problems during the isolation of calf PBECs, since, on one hand, fibroblasts grow faster than epithelial cells and leave limited area for the growth of epithelial cells, and on the other hand elimination of fibroblast contaminations from epithelial cultures are quite challenging. In the current study, stripping and cutting airway epithelium, were two possibilities to minimize the amount of connective tissue in the epithelial cell culture. Stripping epithelium was the most optimal way to improve the quantity and viability of PBECs (Figure 1), probably due to the increase in the efficiency of digestion. Different studies cut the bronchus into smaller fragments and digest these fragments overnight [20], whereas the method described in the current study, stripping epithelium, showed that more than 10 million PBECs can already be harvested after 1h digestion. Fibroblasts can attach very quickly to culture plates, whereas PBECs need 2-3 days to attach [21, 22]. To eliminate these fast-attaching fibroblasts, the cell population was first incubated in a short-incubation medium supplemented with 10% FBS for 1h. Thereafter, serum-free medium (non-FBS) containing essential supplements (**Table 1**) was required for culture and attachment of PBECs [22], resulting in a typical and uniform population of epithelial cells (Figure 2A and B).

In the present study, two different epithelial cell culture systems, including the SCC system and LLI system were optimized for investigating the *M. haemolytica* and LPS-induced inflammation and epithelial dysfunction in PBECs. These cell culture systems are extensively used and described in combination with other primary epithelial cells and cell lines [19, 23, 24].

TLRs are particularly important in recognizing bacterial components in the

airways, where endotoxin (LPS) can be recognized by TLR4 and flagellin can be recognized by TLR5 [25, 26]. Both LPS and flagellin were investigated in PBEC model since they are the main virulence factors of most human-related respiratory bacteria [14]. However, there is not enough experimental evidence that *M. haemolytica,* or its released virulence factors, can induce expression of (epithelial) TLR4 and TLR5. It has been reported that bovine TLRs demonstrate a high sequence similarity with human TLR genes [27, 28]. TLR4 and TLR5 receptors are abundantly expressed in airway epithelial cells of different species, such as cattle, sheep, mice, and humans [14, 26, 28-30]. In the present study, TLR4 and TLR5 protein levels increased 2.5 times and 2 times after stimulation with *M. haemolytica*, respectively, and both the TLR4 agonist (LPS) and TLR5 agonist (flagellin) induced an inflammatory response in PBECs (Figure 4 and Figure S3). It has been reported that the TLR4 mRNA expression increased 6 times and p38, ERK and NF-κB were activated in human airway epithelial cell line stimulated with *Klebsiella pneumoniae* [29]. Another study showed that the TLR5 mRNA expression increased 4 times in human airway epithelial cell line stimulated with *P. aeruginosa* flagellin [26]. Moreover, $TLR4^{-/-}$ mice displayed an attenuated airway inflammatory response induced by *H. influenzae*, which was associated with a significant reduction in the clearance of this pathogen from the respiratory tract [31]. $TLR5^{-/-}$ mice showed inhibition of MAPK phosphorylation and a decrease in inflammatory mediators leading to a reduction of *P. aeruginosa*-induced lung infection. The TLR5–TLR4 cooperation is important for effective immune responses to lung infection [32, 33].

TLR-mediated MAPKs and NF-κB" signaling pathways could play a role in the *M. haemolytica* and LPS-induced inflammation. It is known that the MAPK pathway, including p38, ERK1/2, and JNK, as well as the NF-κB pathway are involved in the regulation of the synthesis of inflammatory mediators [34]. In general, after activation of TLR4 and TLR5, the classical “TLR-mediated MAPKs and NF-κB" signaling cascade will be activated, and NF-κB and MAPK signaling immediately promote the release of cytokines (especially IL-6 and TNF-α) and chemokines (especially IL-8), to recruit immune cells (e.g., macrophages, neutrophils) into the airway to eliminate bacteria [25]. The chemokine IL-8 and cytokines IL-6 and TNF-α have a wide range of effects on different cell types and are involved in the inflammation of respiratory diseases [9, 19, 35-37]. In our study, PBECs stimulated with *M. haemolytica* or LPS showed an increased expression of TLR4 and TLR5, while MAPK (p38, ERK1/2, JNK1/2) and NF-κB p65 were activated, possibly leading to the production of inflammatory cytokines (L-6, TNF-α) and chemokines (IL-8) (Figure 4, 5 and 6). In agreement with our study using undifferentiated PBECs, Cozens *et al.* found that *M. haemolytica* invades

differentiated bovine airway epithelial cells by transcytosis and induces IL-1β, IL-6, TNF-α and IL-8 release by these epithelial cells [37]. It has also been reported that super-infection of human bronchial epithelial cells by *Staphylococcus aureus* leads to an enhanced phosphorylation of the MAPK p38 as well as an increased activation of the MAPKs ERK1/2 and JNK [38]. In addition, *in vivo* and *in vitro* studies showed that *H. influenzae* and *S. pneumoniae* disrupt airway epithelial barrier integrity by stimulating TLR2/4 and by activating the p38 MAPK and TGF-β pathways [39]. Moreover, TLR5 activation in response to bacterial infection may result in impaired epithelial barrier integrity and increased intestinal inflammation [40].

In the present study, the effect of *M. haemolytica* and LPS on the airway barrier function of PBECs was investigated and the cultured PBECs created apical junctional complexes formed by tight and adherens junctions resulting in a tight network after culturing in the LLI system for 8-11 days (Figure 5E, G and H). The peak TEER value was 800 ± 100 Ω·cm^2 measured at day 10 (Figure 5E), which is comparable to the TEER values (700-1200 Ω·cm^2) of human tracheal and bronchial epithelial cells obtained from healthy donors [41, 42]. Cozens *et al.* reported that a peak TEER value from 500 to 1400 Ω.cm^2 was measured in a LLI system with airway epithelial cells derived from different donor calves after approximately 5 days of culture and these cells showed the high intensity of ZO-1 staining after culturing for 11 days in transwells, which is similar to our observations in the LLI system [43]. The air-liquid interface (ALI) culture of human bronchial epithelial cells indicated a maximal TEER value of 766 ± 154 Ω.cm^2 7 days after seeding [44]. In the present study, TEER value dropped to approximately 600 Ω.cm^2 after reaching maximal TEER value at Day 10 (800 ± 100 Ω·cm^2), while at day 11 the TEER value stabilized (Figure 5E). Another study also showed that the TEER of human nasal epithelial cells cultured in a LLI system dropped to approximately 500 Ω.cm^2 after reaching the maximum TEER value (3133 ± 665 Ω.cm^2) [45]. The drop in TEER values before stabilization could be related to the beginning of cell senescence [46] or differentiation state of the cells [43].

When TEER value stabilized (day 11), *M. haemolytica* and LPS were administered to the apical side of the LLI system and a significant decrease of TEER and lower expression of ZO-1 and E-cadherin was observed after *M. haemolytica* and LPS incubation for 24h, which indicated a breakdown of junctional complexes resulting in dysfunction of the bronchial epithelial barrier. In particular, the TEER value started to decrease after 6h *M. haemolytica* stimulation, and after 12h the TEER value mimicked the value observed after seeding the PBECs, which may imply a dramatic disruption of the airway epithelial barrier,

which will promote the rapid invasion of *M. haemolytica* through the epithelium to other tissues and blood vessels, aggravating the severity of acute lung infections. Cozens *et al.* found that the TEER values of bovine airway epithelial cells started to decrease after *M. haemolytica* stimulation for 24h, but a significant decrease occurred after 48h stimulation [37]. Differences between our observations and the study of Cozens et al. could be related to the difference between, the LLI and ALI model, differentiation state of the airway epithelial cells, *M. haemolytica* isolates, and the number of CFUs.

A significant increase in IL-8 release at both apical and basolateral compartments of the transwell inserts was detected after *M. haemolytica* and LPS exposure (Figure 5B). The anti-IL-8 antibody was already be proved to reduce lung epithelial injury and protect the alveolar epithelial barrier after acid-induced lung injury [47, 48]. Moreover, IL-6 and TNF-α release were significantly increased at the apical and basolateral compartments of the transwell insert after exposure to *M. haemolytica* (Figure 5C and D), while LPS only increased the apical IL-6 and TNF-α release by PBECs. In our model, *M. haemolytica* tended to affect the cell viability of PBECs in the LLI system (Figure S4), which may result in lower TEER values (Figure 5E) and higher IL-6 and TNF-α release (Figure 5C and D) after infection. *M. haemolytica* produces besides LPS, also another important virulence factor, leukotoxin, which could also contribute to the observed destructive effects on the airway epithelial layer [17].

Chemokines and cytokines produced by epithelial cells infected with *M. haemolytica* have the ability to recruit and activate cellular innate and adaptive immune cells and regulate airway epithelial barrier function, which can stimulate autophagy, phagocytosis, and clearance of necrotic cells and pathogens, further contributing to the inflammatory responses [9]. Furthermore, the release of IL-8, IL-6 and TNF-α at the basolateral compartment simulate the release of inflammatory mediators from the epithelium into the bloodstream observed in animal and human (childhood) respiratory diseases.

In summary, an optimized culture method for calf PBECs was developed and LPS/*M. haemolytica*-induced inflammatory responses in two different systems (SCC and LLI systems) with PBECs were detected. In addition, a possible mechanism for the induction of cellular inflammation and a decrease of barrier function in epithelial cells induced by *M. haemolytica* or LPS was given. Our data showed that *M. haemolytica* and LPS significantly increased IL-8, IL-6 and TNF-α release and decreased bronchial epithelial barrier function in PBEC, possibly by activating “TLR-mediated MAPKs and NF-κB” signaling pathways (**Figure 6**). Although this is an *ex vivo* model with PBECs from calves, the *M. haemolytica*-

and LPS-induced inflammation, TLR4/5 expression, and lung barrier dysfunction are mimicking important features and mechanisms of respiratory diseases which are frequently observed in other animals and humans (children) from which it is not easy to obtain and culture bronchial epithelial cells. The development of these *ex vivo* models with PBECs will reduce the use of *in vivo* studies and will contribute to the *principles* of Replacement, Reduction and Refinement (*3R's*) [49]. These PBEC culture systems could be used to investigate the pathogenesis and intervention strategies of respiratory diseases.

Methods

Isolation and culture of primary bronchial epithelial cells (PBECs)

PBECs were isolated from bovine lung epithelium obtained from the lungs of freshly slaughtered calves aged 6-8 months provided by Ekro bv (Apeldoorn, The Netherlands). Fresh lungs were always kept on ice during transport to the laboratory. Thereafter, the lungs were washed with PBS (Lonza, Verviers, Belgium) containing penicillin (100 U/mL, Sigma-Aldrich, St. Louis, MO) and streptomycin (100 µg/mL, Sigma-Aldrich) before and after removing the surrounding tissue from the right or left primary bronchus. The bronchus was opened with sterile scissors. [1] The bronchus was cut into smaller pieces or [2] the bronchus was pinned on foam board with sterile needles and bronchial epithelium was carefully stripped from the primary bronchus by using tweezers. For both methods, the similar size and weight of the bronchus was used. The small bronchial pieces [1] or the bronchial epithelium [2] was digested in Dulbecco's modified Eagle's medium (DMEM) containing pronase (1 mg/mL, Sigma-Aldrich), deoxyribonuclease I (500 µg/mL, Sigma-Aldrich), penicillin (100 U/mL, Sigma-Aldrich) and streptomycin (100 µg/mL, Sigma-Aldrich) for 60-90 min at 37 °C and was shaken 3 times during this procedure. Digestion was stopped by adding a triple volume short-incubation medium (**Table 1**). Epithelium debris was removed by passing the tissue through a 70 µm cell strainer (Corning, New York). Cell suspensions were centrifuged at 280 x g for 15 min at 4 °C and resuspended in short-incubation medium (1 x 10^6 cells/mL). These cell suspensions were transferred to 6-well plates (5 mL/well, Corning) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 1h. Supernatants were gently collected and centrifuged at 280 x g for 5 min, resuspended in serum-free medium (**Table 1**) and added to 6-well plates pre-coated with collagen (30 µg/mL, Advanced BioMatrix, San Diego, CA) in combination with fibronectin (10

µg/mL, Sigma-Aldrich), and bovine serum albumin (BSA; 10 µg/mL, Sigma-Aldrich).

Table 1. Primary bronchial epithelial cell culture medium composition.

Composition	Cat. number	Serum-free medium	FBS medium	Short-incubation medium
RPMI-1640	BE12-702F	100%	100%	0%
DMEM	42430	0%	0%	100%
Fetal bovine serum	F7524	0%	10%	10%
Penicillin-streptomycin	P0781	1%	1%	1%
L-glutamine	25030-024	1%	1%	1%
MEM NEAA	11140-035	1%	1%	1%
Sodium selenite	S5261	4 ng/mL	0%	0%
Holo-transferrin	T1283	2.5 µg/mL	0%	0%
Epidermal growth factor	E9644	25 ng/mL	0%	0%
Insulin	I6634	1 µg/mL	0%	0%

Submerged cell culture (SCC) system

PBECs (from *passage* 0) in serum-free medium were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 2-3 days until reaching near-confluence (70-90%) and forming clear network structures. After these 2-3 days, serum-free medium was changed into 10% FBS medium and cells were grown for another 2-3 days.

To subculture the adherent PBECs, PBECs were washed twice with pre-warmed PBS (37 °C), detached and passaged using 0.05% trypsin-EDTA (Gibco, ThermoFisher Scientific, Waltham, MA). The *passage* 1 (P1) of PBECs was centrifuged at 280 x g for 5 min and suspended in FBS medium (**Table I**) in 6- or 96-well plates for the following experiments.

Liquid-liquid interface (LLI) system

P1 of the PBECs suspension (1 x 10^6 cells/mL, FBS medium, 300 µL) was added to the apical compartment of the permeable 0.3 cm^2 high pore density polyethylene membrane transwell inserts (353495, Corning) placed in a 24-well

plate and 700 µL FBS medium was added to the basolateral compartment. The PBECs were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. TEER of PBECs was measured by a Millicell-ERS Volt-Ohm meter (Millipore, Merck, Darmstadt, Germany) every 2 days. The culture medium from the basolateral and apical compartment was refreshed after TEER measurement and experiments started at day 11 when TEER values of about 600 $\Omega \cdot cm^2$ were achieved.

Cell count and viability

Cell counts and differential cell analyses were performed on the PBEC suspension after removing debris by passing through a 70 µm cell strainer. Differential cell counts were determined by Diff-Quick (Medion Diagnostics International Inc., FL, USA) staining on cytospin preparations and a minimum of 500 cells were counted. The viability of PBECs was assessed by trypan blue dye exclusion (0.2%, Sigma-Aldrich, Zwijndrecht, The Netherlands).

Bacterial growth conditions

M. haemolytica (isolated from a pneumonic bovine lung) was kindly provided by Jos van Putten (Utrecht University, The Netherlands). *M. haemolytica* was incubated overnight at 37°C in sheep blood agar (Biotrading, Mijdrecht, The Netherlands).

LPS, flagellin and bacteria *ex vivo* stimulation

P1 of PBECs were cultured at a density of 1 x 10^6 cells/mL in 96- or 6-well plates pre-coated with collagen, fibronectin and BSA or in non-coated transwell inserts. After reaching near-confluence and stable TEER value, these PBECs were stimulated with commercially available LPS (isolated from *E. coli O111:B4*, Sigma-Aldrich) or flagellin (isolated from *P. aeruginosa*, Invivogen, CA, USA) or *M. haemolytica* for 24h and supernatants were collected and stored at -20 °C until analysis.

Lactate dehydrogenase (LDH) assay

P1 of PBECs were grown in 96-well plates or in transwell inserts as described above and the cytotoxic effect of LPS, *M. haemolytica* or flagellin on the PBECs was evaluated by measuring LDH leakage. LDH was measured in the supernatants using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega Corp., Madison, WI, USA) according to manufacturer's instructions.

Thiazolyl blue tetrazolium bromide (MTT) assay

P1 of PBECs were grown in 96-well plates or in transwell inserts as described above and the viability of cells was measured using MTT assay. MTT (Sigma-

Aldrich) was dissolved at a final concentration of 0.5 mg/mL in FBS medium. Each culture well was delicately washed with pre-warmed (37 °C) PBS before adding a 120 µL MTT solution. After 3h incubation (37 °C, 5% CO_2), the formed formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) and absorbance was read at 595 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA).

ELISA measurement

P1 of PBECs were grown in 96-well plates or in transwell inserts as described above. The inflammatory response of PBECs after LPS, *M. haemolytica,* or flagellin stimulation was determined by measuring IL-8 (Mabtech, Nacka Strand, Sweden), IL-6 (Invitrogen, ThermoFisher Scientific) and TNF-α (R&D Systems, Minneapolis, MN) in supernatants using ELISA according to manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories).

Western blotting

P1 of PBECs (1 x 10^6 cells/mL) were incubated with LPS (10 µg/mL) or *M. haemolytica* (1 x 10^5 CFU/mL) at 37°C for 24h in both SCC and LLI systems, thereafter the supernatant was aspirated, and cells were washed 3 times with PBS. After this, total cell lysates were prepared by adding RIPA cell lysis buffer (ThermoFisher Scientific) containing protease inhibitors (Roche Applied Science, Pennsburg, Germany). Total protein content was estimated by bicinchoninic acid (BCA) analysis (Pierce, ThermoFisher Scientific) according to the manufacturer's protocol. 30 µg of protein sample was loaded onto polyacrylamide gels (4-20% Tris-HCl, Bio-Rad Laboratories), separated using electrophoresis, and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). The membranes were blocked with PBS containing 0.05% Tween-20 (PBST) and 5% milk proteins for 1h at room temperature and incubated with primary antibodies at 4 °C overnight (TLR5, 1:250, #sc57461, Santa Cruz Biotechnology, Dallas, TX; TLR4, 1:1000, #pa5-23284, ThermoFisher Scientific; p-p38, 1:1000, #9215; p-ERK1/2, 1:1000, #9101; p-JNK1/2, 1:1000, #9251; p-p65, 1:1000, #3033; β-actin, 1:5000, #4970, Cell Signaling Technology, Beverly, MA; ZO-1, 1:1000, #339100, Invitrogen, ThermoFisher Scientific; E-cadherin, 1:2000, #610182, BD Biosciences), followed by washing blots in PBST. Appropriate horseradish peroxidase-coupled secondary antibodies from Dako (Agilent Technologies, Santa Clara, CA) were applied for 1h. Membranes were incubated with ECL western blotting substrates (Bio-Rad Laboratories) prior to obtaining the digital images. Digital images were acquired with the Molecular Imager Gel Doc XR system (Bio-Rad Laboratories) and analyzed with Image Lab

5.0 (Bio-Rad Laboratories).

Immunofluorescence

P1 of PBECs suspension (1 x 10^6 cells/mL) were grown in 6-well plates (5 mL/well) for 2-3 days until reaching near-confluence (70-90%) or in transwell inserts for 8-11 days until forming stable TEER values as described above. Immunofluorescence staining was conducted to detect the epithelial marker cytokeratin, the tight junction protein ZO-1 and the adherens junction protein E-cadherin. PBECs were fixed with 10% formalin (Baker, Deventer, The Netherlands) and after washing with PBS, the cells were permeabilized with PBS containing 0.1% (v/v) Triton-X-100 for 5 minutes, followed by blocking with 5% serum in 1% (w/v) BSA/PBS for 30 minutes at room temperature. Thereafter, PBECs were incubated overnight with primary anti-wide spectrum cytokeratin antibody (1:50, #ab9377, Abcam, Cambridge, UK), ZO-1 (1:50, #339100, Invitrogen, ThermoFisher Scientific) and E-cadherin (1:50, #610182, BD Biosciences, USA) followed by incubation with Alexa-Fluor conjugated secondary antibodies (Invitrogen, ThermoFisher Scientific) for 1 h at room temperature in dark. Nuclear counterstaining was performed with DAPI containing anti-fade reagent (ready to use, Invitrogen, ThermoFisher Scientific). Cytokeratin, ZO-1 and E-cadherin were visualized, and images were taken using a fluorescence microscope (Keyence BZ-9000, Osaka, Japan). Fluorescence intensity was quantified by ImageJ (Version 1.8.0, National Institutes of Health, USA) and presented as fluorescence intensity (vs DAPI). In addition, cell numbers of cytokeratin were counted by ImageJ and expressed as a percentage of cytokeratin-positive cells (vs DAPI).

Statistical analysis

Experimental results were expressed as means ± SEM. Statistically significant differences between groups were determined by unpaired Student's t-test, one-way or two-way ANOVA using GraphPad Prism (version 7.0). Results were considered statistically significant when $P < 0.05$.

Author contributions

Author contributions: Y.C. and S.B. conceptualized the study; S.B., G.F., S.V., J.P.M.vP. advised on study design; Y.C. directed experiments, analyzed data, performed statistical analysis and wrote the manuscript, S.B., G.F., J.P.M.vP. edited the manuscript. Y.C., S.B., G.F., S.V., J.P.M.vP. gave final approval of the version to be published.

Supplementary information

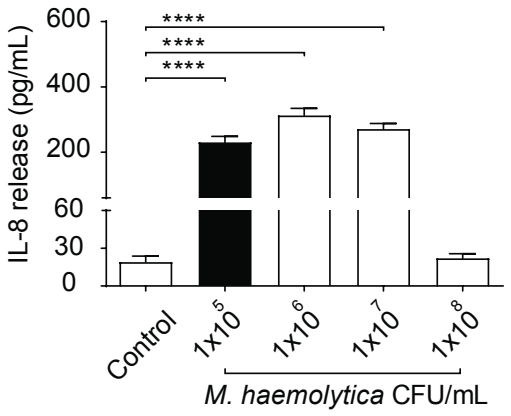


Figure S1. *M. haemolytica* increased IL-8 production by PBECs. *Passage* 1 (P1) of PBECs were incubated with increasing concentrations of *M. haemolytica* (1 x 10^5-10^8 CFU/mL) for 24h in the SCC system and IL-8 levels in the supernatants of control and *M. haemolytica*-treated PBECs were assessed by ELISA. ****P<0.0001 (one-way ANOVA). Data are presented as means ± SEM (n=4 donor calves).

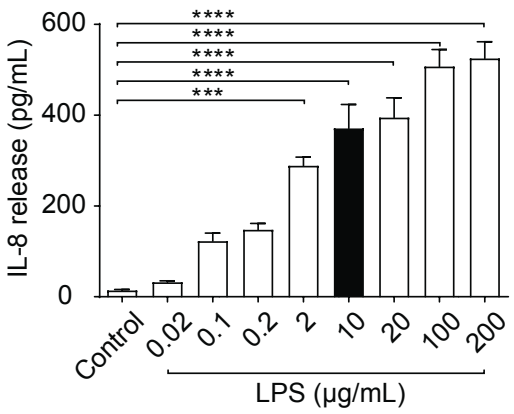


Figure S2. LPS increased IL-8 production by PBECs. *Passage* 1 (P1) of PBECs was incubated with increasing concentrations of LPS (0.02-200 µg/mL) for 24h in the SCC system and IL-8 levels in the supernatants of control and LPS-treated PBECs were assessed by ELISA. ***P<0.001, ****P<0.0001 (one-way ANOVA). Data are presented as means ± SEM (n=3 donor calves).

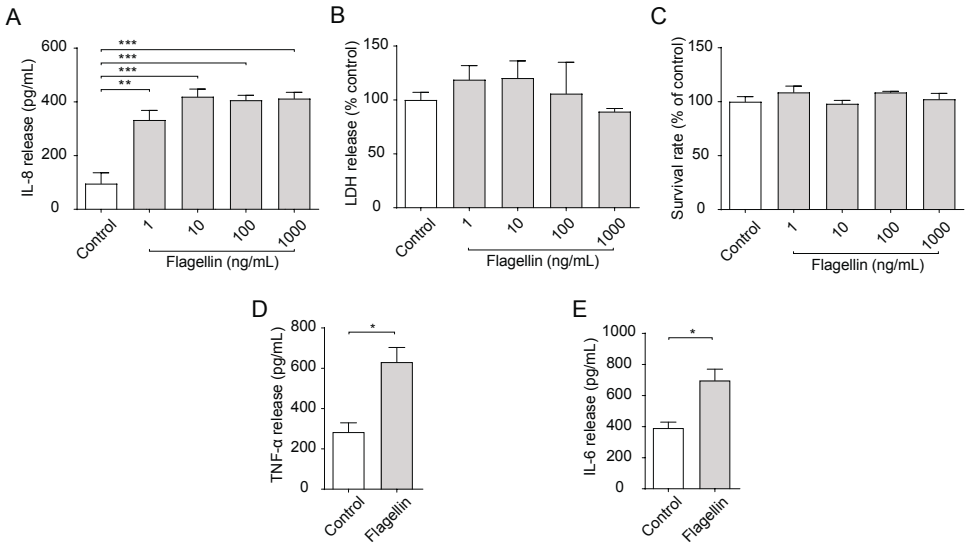


Figure S3. Inflammatory response of PBECs incubated with flagellin. *Passage* 1 (P1) of PBECs were incubated with increasing concentrations of flagellin (1-1000 ng/mL) (**A-C**) for 24h in the SCC system. (**A)** IL-8 levels in the supernatants of control and flagellin-treated PBECs were assessed by ELISA. (**B**) LDH levels were measured in the supernatants and **(C**) survival rates were determined by the percentage of MTT levels in control and flagellin-treated PBECs. (**D-E**) P1 of PBECs were treated with flagellin (10 ng/mL) for 24h in the SCC system. (**D**) TNF-α and (**E**) IL-6 levels in the supernatants of control and flagellin-treated PBECs were assessed by ELISA. **P*<0.05; ***P*<0.01; ****P*<0.001; (**A**–**C**) one-way ANOVA; and (**D-E**) unpaired Student's t-test. Data are presented as means ± SEM (n=3 donor calves).

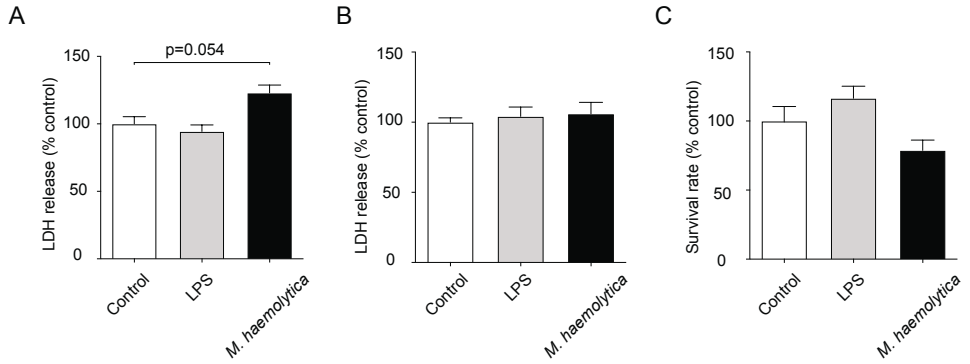


Figure S4. Cytotoxicity effects of *M. haemolytica* and LPS on PBECs. *Passage* 1 (P1) of PBECs were cultured in the LLI system and LPS (10 µg/mL) or *M. haemolytica* (1 x 10^5 CFU/mL) was added to the apical compartment for 24h at day 11. LDH release in the supernatants of the apical (**A**) and basolateral (**B**) compartments were measured and (**C**) survival rates were determined by the percentage of MTT levels in control and LPS/*M. haemolytica*-treated PBECs. *P*=0.054 (one-way ANOVA). Data are presented as means ± SEM (n=3 donor calves).

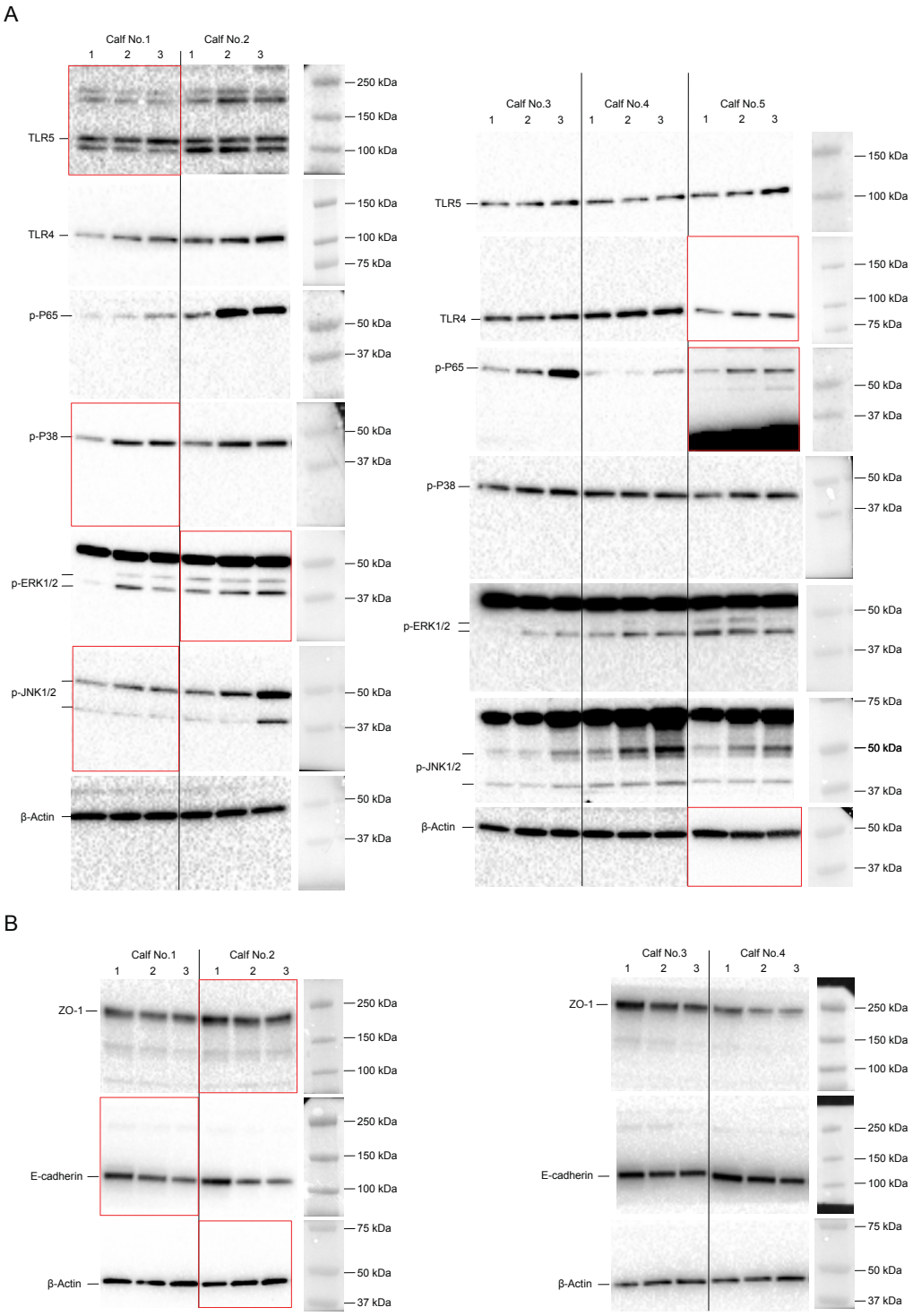


Figure S5. Original blots of figures 4D and 5F.
Lane 1: Control, lane 2: LPS (10 µg/mL) stimulation, lane 3: *M. haemolytica* (1 x 10^5 CFU/mL) stimulation. Figure S5A represents the original blots of figure 4D and figure S5B shows the original blots of figure 5F. The original blots with the red boxes represent the cropped images as shown in figure 4D and 5F.

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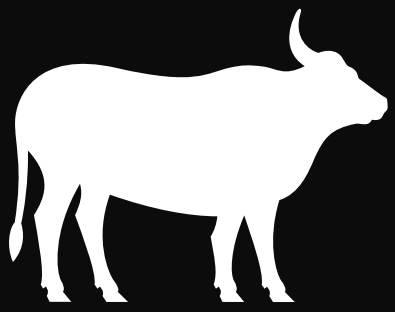

3

Chapter 3

Naturally Occurring Respiratory Infection Model in Calves: Development of Neutrophil-driven Inflammation

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Abstract

Rationale: The calf is one of the species most prone to naturally occurring respiratory infections, in which neutrophils are key players in lung infections. However, excessive neutrophil activation may cause severe tissue damage due to the production of proinflammatory mediators.

Objectives: In this study, the kinetics of immune parameters and the relation between neutrophils and inflammatory cytokines/chemokines have been investigated in naturally occurring respiratory infections.

Methods: Calves were naturally exposed to environmental pathogens from experimental week 0 to 27. Clinical scores were measured every week and rectal temperature was measured from week 1 to 8. Levels of predominant immune cells in blood and bronchoalveolar lavage fluid (BALF) were evaluated at week 0, 2, 4, 6 and 1, 3, 5, 7, respectively. Correlations between neutrophils and chemokine/cytokine levels in BALF and blood were determined. *Mannheimia haemolytica* positivity in BALF was examined over time.

Results: Increased rectal temperature and clinical scores over time indicated the development of respiratory infections in calves. The proportion and number of neutrophils decreased to the lowest level in blood and increased to the highest level in BALF during lung infections, while macrophages decreased to the lowest level in BALF. Interestingly, interleukin (IL)-8 may mediate the recruitment of neutrophils, and IL-6 and tumor necrosis factor-α may induce the activation of neutrophils. The positivity for *M. haemolytica* in BALF increased to the highest level during lung infections.

Conclusions: This study describes the recruitment of neutrophils, depletion of macrophages, production of cytokines/chemokines, and increase of *M. haemolytica* positivity during lung infections in calves, which could supply a valuable model to investigate the pathogenesis and intervention strategies of respiratory infections.

Introduction

Lung infection is one of the leading causes of death in many species, including humans and calves, and especially threatens infants and the elderly [1, 2]. The relation between neutrophils and lung infections has been extensively addressed [3], indicating the important role of neutrophils in the development of infections. Upon lung infection, neutrophils are immediately recruited and traffic from the vasculature to the lung via trans-endothelial migration, after which they arrive in the alveolar space [3]. The recruitment and activation of neutrophils can be initiated and regulated via a multitude of mechanisms, for instance, via cytokines and chemokines produced by lung epithelial cells in response to pathogens [4]. Successful pulmonary immunity requires the rapid response of neutrophils, killing invading pathogens through phagocytosis and the release of anti-bacterial mediators [4]. However, the neutrophil influx into the lungs is a double-edged sword: excessive neutrophil activation can cause severe tissue damage due to the production of proinflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1, and IL-8 [3, 5].

Animal models are widely used to evaluate the efficacy and safety of new vaccine candidates and new therapeutic intervention strategies. Among them, bovine respiratory infections can be a valuable model for studying lung infections in general, due to the high natural prevalence, easy-to-observe respiratory symptoms, and the continuous production of inflammatory mediators and neutrophils in the airways [2, 6]. Calves are one of the species with a high prevalence of naturally occurring respiratory infections, including lung infections, in which *M. haemolytica* has been claimed to be one of the major opportunistic bacteria [7]. Although bovine respiratory diseases have been described for decades [8, 9], limited information is available on the kinetics of immune and inflammatory parameters and specific disease characteristics.

Here, we investigated the relation between leukocytes and inflammatory cytokines/chemokines in bronchoalveolar lavage fluid (BALF) and blood of calves during natural exposure to environmental pathogens. Our data indicate that a large number of neutrophils enter the lungs from the blood vasculature during lung infections. Furthermore, the influx of neutrophils might be related to the increased cytokine/chemokine (e.g., TNF-α, IL-6, and IL-8) levels in BALF and/or blood of calves with lung infections.

Results

Experimental timeline and the development of clinical symptoms

The experimental design and antibiotic treatments are displayed in **Figure 1A**. During the experimental stage of 50 calves, clinical scores, a scoring system for clinical symptoms including coughing, nasal discharge, and behavior, were measured every week. Rectal temperature was measured from week 1 to 8. Compared with week 1 (the first week when calves arrived at the facilities), the rectal temperature and clinical scores significantly increased over time, illustrating the development of respiratory infections (**Figure 1B** and **C**). From week 1 to week 6, a rapid increase in the rectal temperature and clinical scores was observed, until they reached the peak for the first time at week 6 (**Figure 1B** and **C**). After that, the clinical scores varied between score 1.0 to 1.5 from week 8 to 27 (**Figure 1C**).

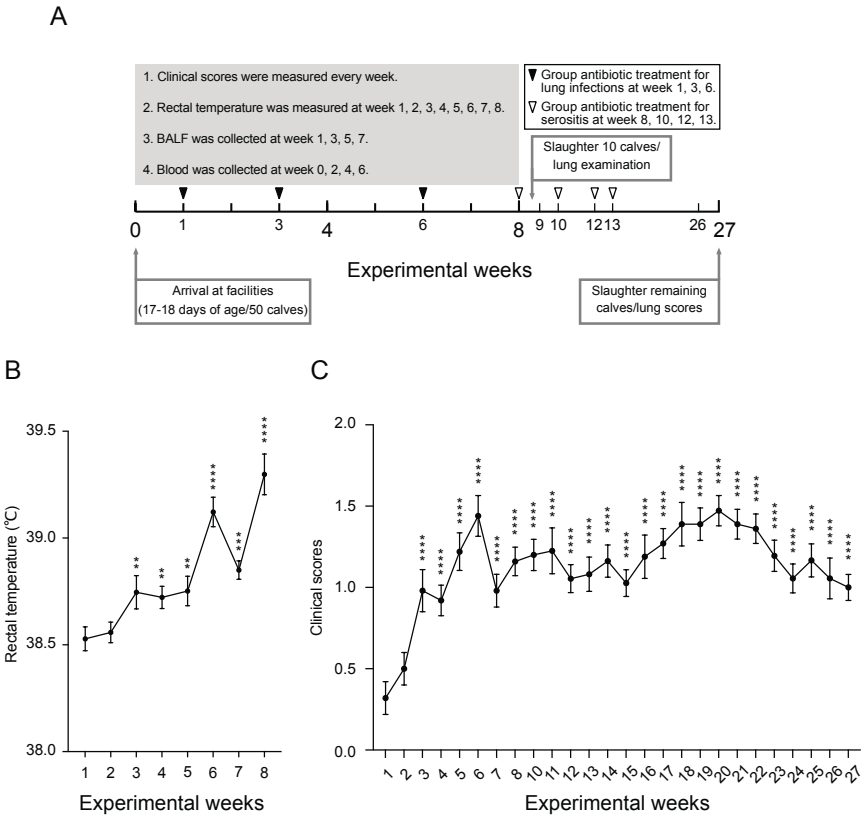

Figure 1. Experimental timeline and the development of clinical symptoms. (**A**) Timeline and design of the experiment. (**B**) Rectal temperature was measured at week 1, 2, 3, 4, 5, 6, 7, and 8 (n=50 calves). (**C**) Clinical scores including coughing, nasal discharge, and behavior were evaluated every week (n=40-50 calves). ***P*<0.01; ****P*<0.001; *****P*<0.0001 (week 2-27 vs week 1). Data are presented as means ± SEM.

Changes of predominant immune cells in BALF and blood during the development of naturally occurring respiratory infections

Respiratory infections of calves usually occur within two months of their arrival at the facility. To investigate the changes of leukocytes, blood was collected at week 0, 2, 4, and 6, and BALF was collected at week 1, 3, 5, and 7 (**Figure 1A**).

At baseline (week 0), the main types of leukocytes in blood were neutrophils and lymphocytes, consisting of 64% and 32% of the total number of leukocytes, respectively, while monocytes equaled 1% (**Figure 2A** and **B**). At week 2, the proportion of neutrophils decreased to 39%, and lymphocytes and monocytes increased to 52% and 6%, respectively (**Figure 2A**). At week 4, the proportion of neutrophils further decreased to 29%, and lymphocytes and monocytes further increased to 55% and 13%, respectively (**Figure 2A**). At week 6, the proportion of neutrophils decreased to 39%, and the proportions of lymphocytes and monocytes increased to 48% and 11%, respectively (**Figure 2A**). The changes in concentration were consistent with changes in the proportion of leukocytes in blood (**Figure 2B**). Neutrophils decreased to the lowest concentration at week 4, while increased at week 6 (**Figure 2B**). Lymphocytes and monocytes increased to the highest number at week 2 and week 4, respectively, and remained stable thereafter (**Figure 2B**). In addition, the proportions and concentrations of eosinophils and basophils remained low and increased slightly at week 2 (**Figure 2A** and **B**).

The main cell types in the BALF were macrophages, neutrophils, and lymphocytes (**Figure 2C** and **D**). At week 1 (the first week after calves arrived at facilities), the proportions of macrophages and neutrophils in BALF were approximately 83% and 17%, respectively, while lymphocytes only represented 0.03% (**Figure 2C**). At week 5, macrophages decreased to 30%, and neutrophils increased to 69%, while lymphocytes slightly increased to 0.6% (**Figure 2C**). At this moment, neutrophils replaced macrophages as the main cell type. At week 7, the proportion of macrophages decreased to 41%, and the proportions of neutrophils and lymphocytes increased to 58% and 0.4%, respectively, but neutrophils were still the main cell type in BALF (**Figure 2C**). The changes in absolute numbers were consistent with changes in the proportion of BALF cell numbers (**Figure 2D**). Neutrophils and lymphocytes increased to the highest number at week 5, and slightly decreased at week 7, while macrophages decreased to the lowest number at week 5 (**Figure 2D**).

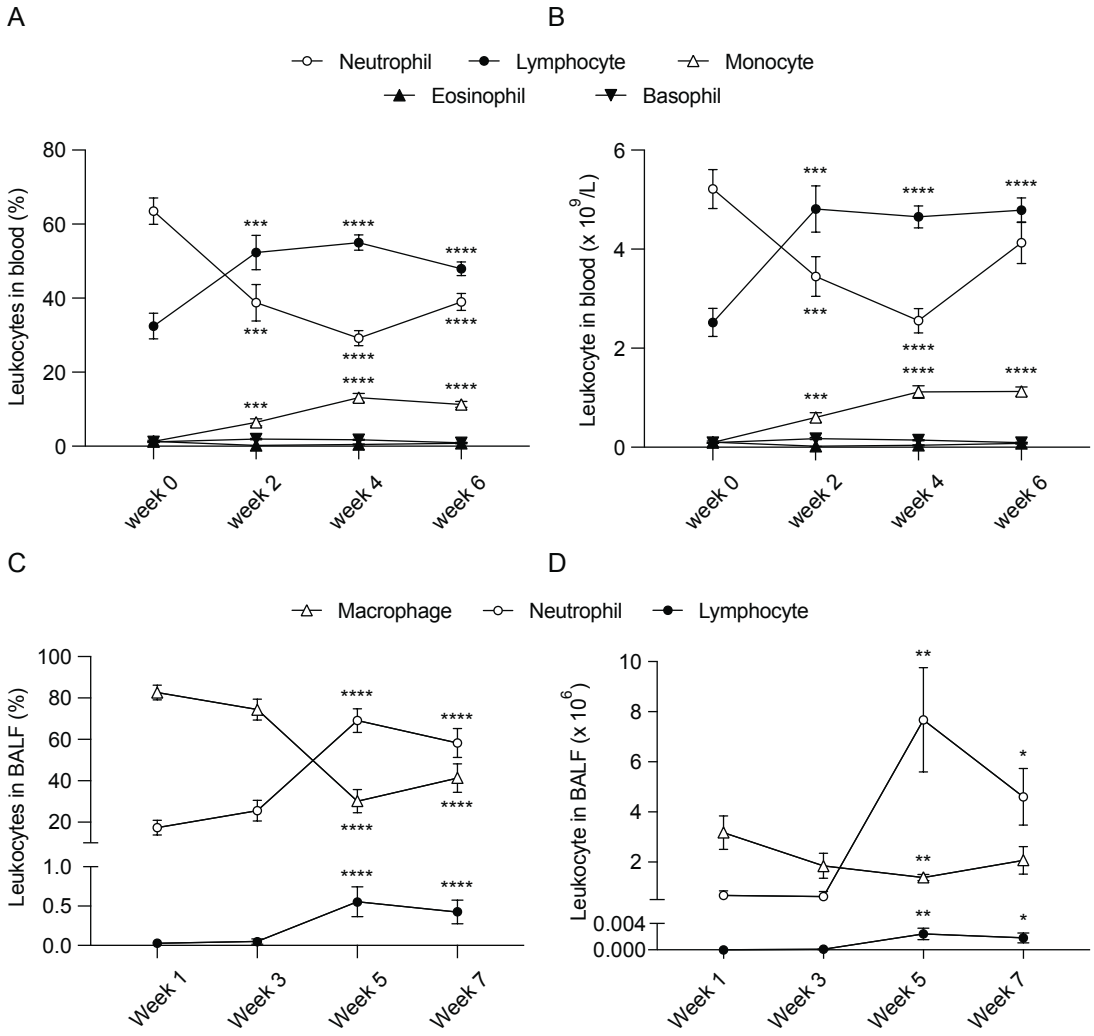

Figure 2. Changes of predominant immune cells in BALF and blood during the development of naturally occurring respiratory infections. (**A-B**) Percentage and concentration of neutrophils, lymphocytes, monocytes, eosinophils, and basophils in blood were measured at week 0, 2, 4 and 6 (n=20 calves). (**C-D**) Percentage and number of macrophages, neutrophils, and lymphocytes in BALF were measured at week 1, 3, 5 and 7 (n=20 calves). ***P<0.001; ****P<0.0001 (week 2-7 vs week 0 or 1). Data are presented as means ± SEM.

Concentrations of IL-8, TNF-α, IL-6, and IL-1β in BALF and blood during lung infections

The presence of lung infections was observed in these calves, possibly from week 4 to 6, since (1) the proportion and number of lymphocytes (number increased by 0.85-fold) and monocytes (number increased by 11-fold) increased

to the highest level, and neutrophils (number decreased by 0.51-fold) decreased to the lowest level in blood at week 4; (2) the proportion and number of neutrophils (number increased by 11-fold) and lymphocytes (number increased by 138-fold) increased to the highest level, and macrophages (number decreased by 0.56-fold) decreased to the lowest level in BALF at week 5; (3) the clinical score continued to increase until the first peak at week 6 (increased by 3.5-fold).

To investigate the inflammatory response, the levels of cytokines and chemokines were measured in blood and BALF during these lung infections. Compared with week 4, the levels of IL-8 and TNF-α significantly increased to 2394 pg/mL (vs 37 pg/mL) and 1518 pg/mL (vs 655 pg/mL) respectively in blood at week 6, while the levels of IL-6 and IL-1β slightly decreased to 811 pg/mL (vs 939 pg/mL) and 58 pg/mL (vs 246 pg/mL), respectively (**Figure 3A**). In addition, the levels of IL-8, TNF-α, IL-6, and IL-1β in BALF at week 5 were on average 93, 904, 1539, and 507 pg/mL, respectively (**Figure 3B**).

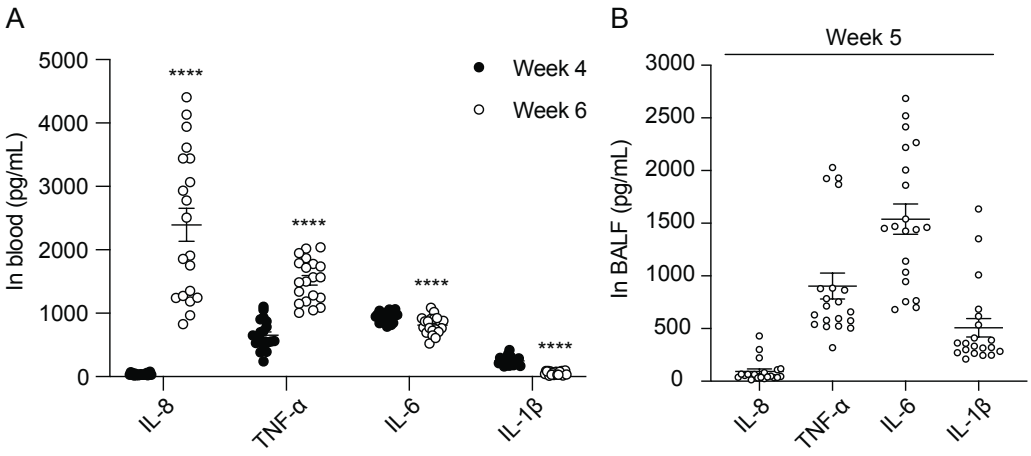

Figure 3. Concentrations of IL-8, TNF-α, IL-6, and IL-1β in BALF and blood during lung infections. (**A**) IL-8, TNF-α, IL-6, and IL-1β concentrations in blood were determined by ELISA at week 4 and 6 (n=20 calves). (**B**) IL-8, TNF-α, IL-6, and IL-1β concentrations in BALF were measured by ELISA at week 5 (n=20 calves). ****P<0.0001 (week 6 vs week 4). Data are presented as means ± SEM. Each dot represents one calf at a single timepoint.

Correlations between neutrophils and chemokine/cytokine levels in BALF and blood during lung infections

The correlations were analyzed to investigate the relation between neutrophils and inflammatory cytokines/chemokines in BALF and blood. Possible negative correlations were found between the concentrations of neutrophils and IL-8 in blood at week 4 (r= -0.37, p= 0.1; **Figure 4A**). Possible positive correlations were observed between the concentrations of neutrophils and IL-6 in blood at week 6 (r= 0.38, p=0.1; **Figure 4B**). Significant positive correlations were measured between the number of neutrophils and the concentrations of TNF-α in BALF at week 5 (r= 0.75, p<0.001; **Figure 4C**).

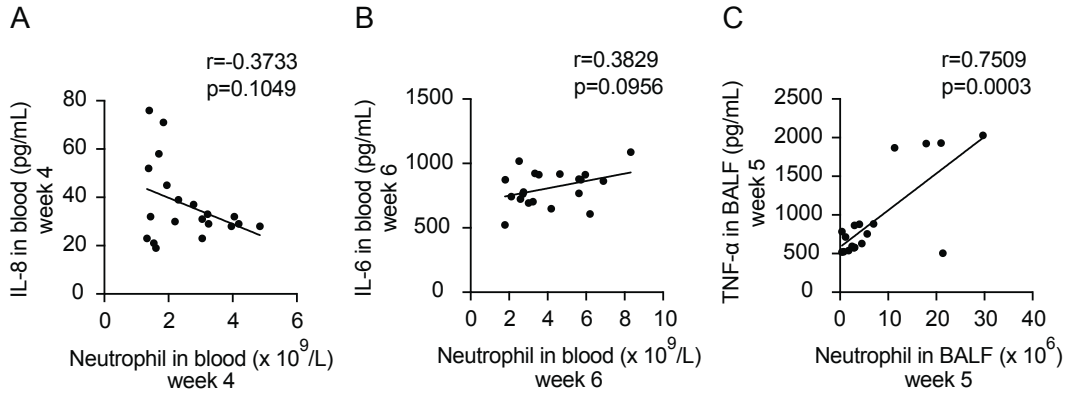

Figure 4. Correlations between neutrophils and chemokine/cytokine levels in BALF and blood during lung infections. (**A**) Pearson correlations between the concentrations of neutrophils and IL-8 in blood at week 4. (**B**) Pearson correlations between the concentrations of neutrophils and IL-6 in blood at week 6. (**C**) Pearson correlations between the number of neutrophils and the concentrations of TNF-α in BALF at week 5.

M. haemolytica in BALF

M. haemolytica is one of the main bacteria that contributed to lung infections in calves [10]. To determine the positivity for *M. haemolytica* in these calves, the *M. haemolytica*-LPS IgG levels were investigated in BALF over time. Compared with week 1 (0%), the positivity for *M. haemolytica* in BALF increased to the highest level (80%) at week 5 and 7, indicating *M. haemolytica* was present in the lower respiratory tract of most calves at week 5 and 7 (**Table 1**).

Table 1. The number and percentage of calves positive for *M. haemolytica* in BALF over time.

BALF samples (n=20 calves/group)	*M. haemolytica*-LPS lgG positivity	*M. haemolytica* positivity (%)	*p-value* (vs week1)
Week 1	0	0	—
Week 3	4	20	0.11
Week 5	16	80	< 0.0001
Week 7	16	80	< 0.0001

BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharides.

Proportion of different lung lesions

Healthy lungs show pale orange color with no sign of pneumonia (**Figure 5A**), while pneumonic lungs have several spots of grey-red discoloration or even the presence of abscesses (**Figure 5B**). At week 8, 10 calves were dissected, which allowed evaluation of the severity of lung infections. Eight out of 10 calves had pneumonia of varying severity, of which moderate pneumonia was the most prevalent (40%) (**Figure 6A**). In addition, the lesions were more observed in cranial and cardiac lobes of these calf lungs.

Four calves died during week 9 to 27, and the remaining 36 calves were slaughtered after 27 weeks, and their lungs were examined to determine the long-term presence of the infection or scars after infection (**Figure 1A**). After 27 weeks, 67% (24/36) of calves still had different stages of lung lesions, of which moderate lesions were most prevalent (31%) (**Figure 6B**).

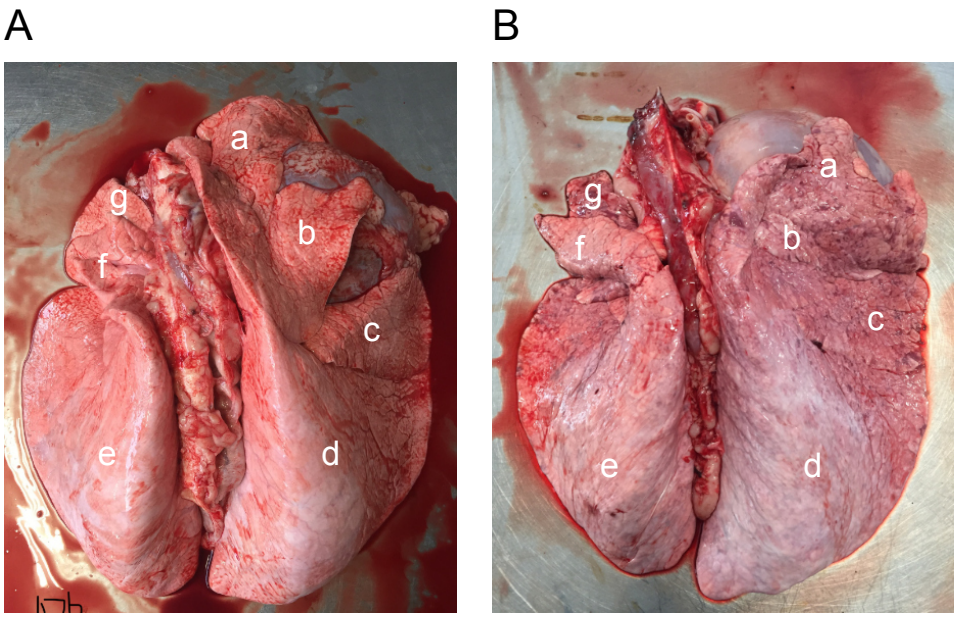

Figure 5. Healthy (A) and infected (B) calf lungs (lobes). a and b = RCrL (right cranial lobe); c = RCaL (right cardiac lobe); d = RDL (right diaphragmatic lobe); e = LDL (left diaphragmatic lobe); f = LCaL (left cardiac lobe); g = LCrL (left cranial lobe).

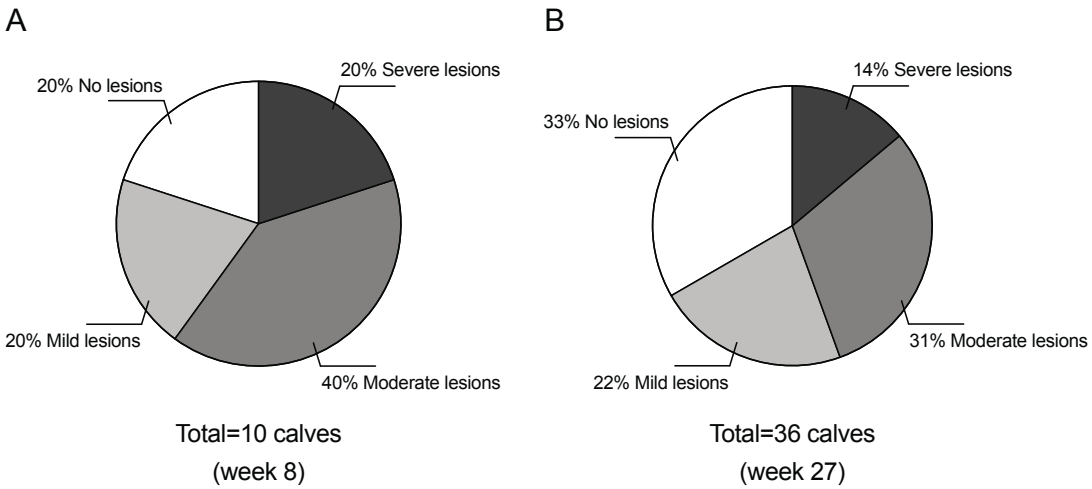

Figure 6. Proportion of different lung lesions. Proportion of different lung lesions was determined based on the lung scores in (**A**) the selected 10 calves at week 8 and in (**B**) the remaining 36 calves at week 27.

Discussion

The goal of this study was to describe the kinetics and (clinical) disease characteristics of parameters related to naturally occurring respiratory infection in calves. Our data indicate that the lung infections have developed within (at least) 8 weeks after the calves arrived at the facility. This is mainly due to that these calves originate from a very diverse background (i.e., different farms) and these calves are transported under stressful conditions.

Clinical symptoms of respiratory infections in calves include fever, cough, nasal discharge, head tilt (headache), lethargy, and decreased appetite [11]. Most of these symptoms are not easy to observe in rodents, for instance, mice and rats do not exhibit an easy-to-observe cough reflex. In the present study, the clinical scores and rectal temperature of calves increased considerably within 6 weeks, and the first peak value occurred at week 6. This may be due to the stress from transportation and the new environment/food; calves are clearly at the highest risk of infection between 2-6 weeks after transport/arrival [12, 13]. At week 7, there is a slight drop in the clinical scores and rectal temperature of the calves, which might be related to the antibiotic treatment against lung infections throughout week 6. From week 7 to week 20, the clinical scores increased slowly and began to decrease continuously after week 20. Although calves received group antibiotic treatments at week 1 and 3 for (metaphylactic) treatment after arrival, which might postpone the peak occurrence of respiratory infections, it did not prevent infections in these calves. The changes in the microbiota of the gastrointestinal and respiratory tract and the increase of drug-resistant pathogens caused by antibiotics might be the reasons for the failure of the prevention and treatment of respiratory infections [14]. However, our data showed that clinical symptoms of respiratory infection persist to 27 weeks, which may represent the development of chronic (recurrent) lung infections. This observation was supported by lung scores, 67% of the calves still had lung lesions of different severity at week 27. In addition, chronic respiratory diseases, including (poly)serositis, which occurred quite persistently after week 9 (data not shown), might also contribute to the persistence of clinical symptoms. Due to the presence of serositis in calves, antibiotic treatments against serositis have been applied at week 8, 10, 12, and 13.

The notable recruitment of neutrophils to the lung was observed in the present study. Neutrophils are abundant in the airways and lungs of mice [15], calves [16], and humans [17] during respiratory infections. For example, in infants with severe respiratory bronchiolitis, neutrophils accounted for > 90% of the BALF composition [17]. In the current study, the neutrophils in blood decreased to the

lowest level at week 4, and this coincided with an increase in neutrophils in BALF to the highest level at week 5. The changes of neutrophils in blood appeared to be negatively correlated with those in BALF, indicating the infection drives the recruitment of neutrophils from the blood to the lungs. To recruit neutrophils, pathogens must trigger and initiate the production of proinflammatory mediators and neutrophil chemoattractants, of which IL-8 is the most representative chemokine [18]. The possible negative correlations between the concentrations of IL-8 and neutrophils in blood at week 4 indicated that IL-8 may drive the decrease of neutrophils in blood. Moreover, IL-8 remained at a low level in blood at week 4 and in BALF at week 5 but increased 65-fold in blood at week 6, which indicated neutrophil recruitment in blood and hence in the lungs. Excessive neutrophil accumulation can mediate inflammatory damage in the lungs [19]. In order to exert the full antimicrobial functions for host defense, neutrophils must be activated by multiple signals, including cytokines such as TNF-α, IL-6 and IL-1β, which can not only recruit neutrophils to the lesions but also drive neutrophil activation [18, 20]. Indeed, a limited difference of TNF-α, IL-6 and IL-1β concentrations in blood and BALF were observed during the lung infections. In addition, the positive correlations between the levels of TNF-α and neutrophils in BALF at week 5 and the possible positive correlations between the concentrations of IL-6 and neutrophils in blood at week 6 indicated that there might be an activation of neutrophils by IL-6 and TNF-α. Neutrophil activation is an event that occurs already during recruitment in the blood [18, 20].

Macrophages are other predominant immune cells and the main resident phagocytes in the lungs under healthy conditions [21]. A small amount of the macrophages in the lung are derived from self-replication, and the rest originate from monocytes recruited from the blood [22]. In addition to phagocytosing pathogens, macrophages are also responsible for the regulation of the inflammatory response and clearance of apoptotic neutrophils [22]. However, due to the aggravation of lung infections, the number of macrophages after phagocytosis of pathogens, apoptotic cells and cell debris is reduced and cannot be replenished in time, causing depletion. Macrophage depletion can result in a failure to control the inflammatory response with increased numbers of neutrophils and proinflammatory cytokines and a decrease in clearance of apoptotic neutrophils [23]. These apoptotic neutrophils then become secondarily necrotic neutrophils, leading to lung injury and organ dysfunction and even death [22, 24]. In the current study, neutrophils were increased at week 5 and 7 in BALF, while macrophages decreased and remained at a low level, indicating a depletion of macrophages in the lungs. Although the monocytes in blood slightly increased at week 4 and 6, it was not sufficient to supplement the depletion of macrophages

due to infection and inflammation in the lungs.

The lymphocytes increased in blood and BALF in the present naturally occurring respiratory infections. Although lymphocytes do not account for a high proportion in BALF, they may represent the presence of co-infections of bacteria and virus(es) in these calves [25]. It is well known that in presence of viral co-infection, residents of the upper respiratory tract (opportunistic bacteria) are more likely to invade into the lower respiratory tract to induce bacterial pneumonia [2, 12].

Although the host can induce an orchestrated immune response to acute lung infections, lung injury is inevitable when the response is overwhelmed by infection [2]. The lung scores indicated that a considerable proportion of the lungs had moderate to severe lung lesions, which represents the failure of lung defense in most calves. In addition, the failure of group antibiotic intervention highlights the importance of developing new intervention strategies. Of course, 20-33% of the calves did not show lung lesions, which is also observed in (inoculated) calf model of lung infections and in the pattern of human respiratory infections, clinically healthy or asymptomatic individuals are always present [26-28]. In addition, the lesions were more observed in cranial and cardiac lobes of the calf lungs, which are determined by the anatomical characteristics.

The main *Pasteurellaceae* causing bacterial pneumonia in calves is a group of well-characterized opportunistic bacteria, including *M. haemolytica, H. somni* and *P. multocida* [2]. *M. haemolytica* has traditionally been the most common bacterial isolate in calves [12]. These bacteria are residents of the upper respiratory tract of calves and invade into the lower respiratory tract with the stimulation of cofactors (e.g., virus, stress, cold air), thereby growing explosively [2, 12]. In the present study, the positivity for *M. haemolytica* in the lower respiratory tract increased from 0% to 80% within 5 weeks, reflecting the invasion of opportunistic pathogens. However, more in-depth microbiological analysis is required to determine the relationship between *M. haemolytica* and lung infections in the present study. Although this calf model cannot compare to rodent models with respect to 1) genetic manipulation, 2) ease of experimentation, and 3) the wide availability of reagents [2], it can provide rapid and reproducible respiratory sampling (e.g., BALF sampling biweekly) and measurements in living organisms under natural conditions. Overall, this calf model can contribute to a better general understanding of the processes and pathogenesis of respiratory infections.

In conclusion, our results demonstrate the recruitment of neutrophils, depletion of macrophages, production of inflammatory cytokines/chemokines and

increase of *M. haemolytica* positivity during lung infection in calves. These parameters can contribute to the diagnosis of (subclinical) bovine respiratory diseases. The failure of the respiratory defense in reaction to acute lung infections highlights the importance of new intervention strategies. In addition, we provide a natural exposure model in calves to investigate the pathogenesis and intervention strategies of respiratory infections.

Materials and Methods

Animal experiment design

This experiment was conducted under the Dutch Law on Animal Experiments in accordance with EU Directive 2010/63 at the research facilities of the VanDrie Group (Scherpenzeel, The Netherlands) and was approved by the Animal Care and Use Committee of Wageningen University (AVD1040020185828, Wageningen, The Netherlands).

The experiment described in this article was part of a large calf trial, including 300 male Holstein Friesian calves (~18 days of age) randomly distributed into a control group and 5 other groups with different (dietary) interventions. In accordance with the purpose of this chapter, we reported here the results of the analyses of the control group. The control group had 50 calves and the experiment started when calves arrived at the experimental facilities.

During the experiment, all calves were naturally exposed to pathogens in the environment. An antibiotic strategy was applied in this large calf trail, and this was the same for all groups (the control group and five intervention groups). Individual antibiotic treatment was applied when required based on clinical signs of illness as assessed by the animal caretakers. The number of applied individual antibiotic treatments did not differ between the control and intervention groups ($P > 0.1$). Group antibiotic treatment was applied equally to all groups (300 calves) if 10% of the calves had been treated within 5 days, or if 5% of the calves had become ill within 24h or when the situation required group antibiotics in the expert judgement of a veterinarian.

From experimental week 1 to 8, blood and BALF were collected bi-weekly. At the end of week 8, 10 calves were slaughtered for lung examination. At week 27, all remaining calves were slaughtered, and lungs were scored. Measurements were performed for all calves or for a subset of calves. The subset of calves included 2 calves per pen and 20 calves in total and was selected on body weight at arrival, closest to the average body weight of all calves at arrival.

Animals, housing and feeding

Calves were housed in a mechanically ventilated stable throughout the experiment. The ambient lighting consisted of natural lighting plus artificial lighting from 0600 to 1800 h. Calves were housed in pens (9 m^2) containing wooden-slatted floors. In the first 6 weeks after arrival, individual housing was applied (1.2 m^2/calf) by placing stainless steel fences within the pens. After 6 weeks, the individual fencing was removed, and calves were housed in groups of 5.

Fifty male Holstein Friesian calves (43.6 ± 0.45 kg, means ± SEM) of German origin were fed with calf milk replacer (MR) twice a day and solid feed (composed of muesli and chopped wheat straw) according to a practical feeding scheme. The MR in the first 8 weeks contained 527 g/kg whey powder, 35 g/kg lactose, 52 g/kg delactosed whey powder, 50 g/kg whey protein concentrate, 60 g/kg soy protein concentrate, 50 g/kg soluble wheat protein, 3 g/kg pea fiber, 179.4 g/kg fat sources, 9.7 g/kg calcium formate, 2 g/kg citric acid, 4 g/kg sodium bicarbonate, 3.5 g/kg mono ammonium phosphate, 9.8 g/kg lysine, 2.4 g/kg methionine, 1.3 g/kg threonine, 0.2g/kg aroma and 10 g/kg premix. The nutrient of MR contained 231 g/kg crude protein, 193 g/kg crude fat and 453 g/kg lactose.

The MR fed after week 8 contained 560 g/kg whey powder, 93 g/kg delactosed whey powder, 55 g/kg soy protein concentrate, 41 g/kg soluble wheat protein, 169 g/kg fat sources, 10 g/kg calcium formate, 2 g/kg citric acid, 4 g/kg sodium bicarbonate, 2 g/kg mono ammonium phosphate, 8 g/kg lysine, 3 g/kg methionine, 2 g/kg threonine, and 10 g/kg premix. This nutrient of MR contained 191 g/kg crude protein, 184 g/kg crude fat and 455 g/kg lactose.

Clinical symptoms

Clinical symptoms were investigated weekly for all calves, adapting from the Wisconsin calf respiratory scoring system [11]. Rectal temperature was measured weekly from experimental week 1 to 8. Moreover, a score from 0 to 3 was provided for coughing, nasal discharge and behavior from week 1 to 27 according to **Table 2**. When evaluating the entire experiment (week 1-27), clinical score was calculated as the sum of these 3 scores, while rectal temperature was displayed separately from experimental week 1 to 8.

Table 2. Clinical parameters and point scale used for respiratory clinical scoring.

Clinical parameter	Points and description			
	0	1	2	3
Rectal temperature (°C)[1]	< 38.5	38.5 - 38.9	39.0 - 39.5	≥ 39.5
Coughing score	None	Single cough	Induce repeated coughs or occasional spontaneous	Repeated spontaneous coughing
Nasal discharge score	Normal, slightly serous	Small amount of unilateral, cloudy discharge	Bilateral, cloudy, or excessive mucus	Copious, bilateral mucopurulent nasal discharge
Behavior score	Normal	Slight lethargy, lies down longer	Lethargy, mostly lying down	Strong lethargy, does not stand up

[1]Rectal temperature was included in clinical scores when evaluating the experimental period (week 1-8) and excluded in clinical scores when evaluating the entire experiment (week 1-27).

Lung scores

Calf lungs were scored and obtained by using a scoring system adapted from Leruste *et al.* [29]. Briefly, the observer (veterinarian) visually examined each lung (cranial and ventral lobes) evaluating signs of pneumonia. Each examined lung was classified according to a 4-point scale for pneumonia from healthy lung (score 0) to severe lesions (score 3). Score 0 for healthy lungs (pale orange color with no sign of pneumonia), score 1 for minimal or mild lesions (one spot of grey-red discoloration), score 2 for moderate lesions (one larger or several small spots of grey-red discoloration with a total surface of less than 1 lobe), and score 3 for severe lesions (grey-red discoloration area of at least one full lobe and/or presence of abscesses). The results were shown as a percentage of the total calves with different severity of pneumonia.

Blood sampling and hematological analyses

Blood samples were collected of all calves by venipuncture in the jugular vein at arrival before the first MR feeding (baseline, week 0), and additionally at experimental week 2, 4 and 6 from 20 calves per group. Blood was collected in 9 mL and 4 mL K_2-EDTA tubes and was kept on ice for collection of plasma or kept at room temperature for analysis of leukocyte numbers the same day by

fluorescence flow cytometry using a Sysmex 1800iV (Sysmex Europe GmbH, Norderstedt, Germany), respectively. Plasma was collected after centrifugation at 2,000 x *g* and 4°C for 20 min and was stored at -20°C pending further analyses (cytokine/chemokine measurement by ELISA).

BALF sampling and phenotyping

BALF samples were obtained by use of a technique adapted from a previous description [30]. Briefly, a calf was restrained in the feeding fence and the head of calf was lifted and extended so that the nasal bone was parallel to the ground. Ethanol (70%) was used to clean the nose/nostrils of the calf. A sterilized 100 cm BAL catheter was then inserted through a naris and blindly guided through the nasal passage into the trachea until the end was wedged in a bronchus. The correct placement of the catheter was verified by elicitation of the coughing reflex, the outstretch of the tongue, movement of air into and out of the catheter with each breath, and the absence of rumen contents, odor, and gurgling from the catheter. Once wedged in the appropriate location, a syringe was connected to the catheter and a total of 30 mL of sterile saline (0.9% NaCl, 37°C) solution was infused into the tube and fluid was immediately aspirated from the bronchus. BALF (18.5 ± 0.39 mL, mean ± SEM) was obtained from each calf and was stored in a 50 mL tube on ice until further processing in the lab the same day.

After transport and arrival at the lab, the volume of BALF was recorded and BALF suspension was filtered by passing through a 70 µm cell strainer (Corning, NY, US) to remove debris. To obtain cell pellets and perform cell counts, BALF suspension was centrifuged (5 min, 400 x *g* at 4°C) and the remaining pellet was re-suspended in 1 mL cold FCS (4°C). After centrifugation, the supernatant was aliquoted into 1.5 mL tubes and stored at -80°C for further analysis. One aliquot was used to determine the cell number by automatically counting in Cellometer Bright Field cell counter (Nexcelom Bioscience, Lawrence, MA). After counting, 0.5 x 10^6 cells of BALF suspension were used to make cytospins and phenotyping were determined by Diff-Quick (Medion Diagnostics, Medion Diagnostics International Inc., Miami, FL) staining on cytospin preparations and a minimum of 400 cells were counted.

Measurement of *M. haemolytica* positivity in BALF

BALF were collected and stored as described above. To investigate the positivity of *M. haemolytica*, *M. haemolytica*-LPS IgG levels were detected in BALF according to manufacturer's instructions (BIO/K-139, Bio-X Diagnostics, Rochefort, Belgium).

ELISA measurement

Levels of IL-8 (Mabtech, Nacka Strand, Sweden), IL-6 (Invitrogen, ThermoFisher Scientific, Waltham, MA), IL-1β (Invitrogen) and TNF-α (R&D Systems, Minneapolis, MN) in the BALF and blood of calves were determined by using ELISA kits according to manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA).

Statistical analysis

Experimental results are expressed as non-transformed means ± SEM and analyzed for time effects with SAS 9.4 (SAS Institute Inc., Cary, NC), using the MIXED procedure, including time as a repeated statement with calf as unit. For each parameter, the covariance structure was selected based on the lowest AIC and BIC. All analyses included a random effect of pen. For blood leukocyte concentration, the concentration at arrival was included as a co-variable in the model. Studentized residuals of each model were checked visually on the homogeneity of variance and data were transformed if required to obtain homogeneity of variance. The effect of time on clinical scores was assessed with the estimate statement, using the GLIMMIX procedure with a multinomial distribution including a random pen effect. Pearson's correlations were applied for the relations between neutrophils and inflammatory cytokines/chemokines in BALF and blood. A Chi-square test was performed for the positivity of *M. haemolytica* in calves. Differences were considered significant when $P < 0.05$.

Acknowledgements: This research was performed in the public-private partnership 'CarboKinetics' coordinated by the Carbohydrate Competence Center (CCC, www.cccresearch.nl). CarboKinetics is financed by participating industrial partners Agrifirm Innovation Center B.V., Cooperatie AVEBE U.A., DSM Food Specialties B.V., FrieslandCampina Nederland B.V., Nutrition Sciences N.V., VanDrie Holding N.V. and Sensus B.V., and allowances of The Netherlands Organisation for Scientific Research (NWO).

Funding information: This work was funded by the Netherlands Organisation for Scientific Research (NWO), grant number: ALWCC.2015.4. Research grant funding (201608320245) was received from the China Scholarship Council for Y. Cai.

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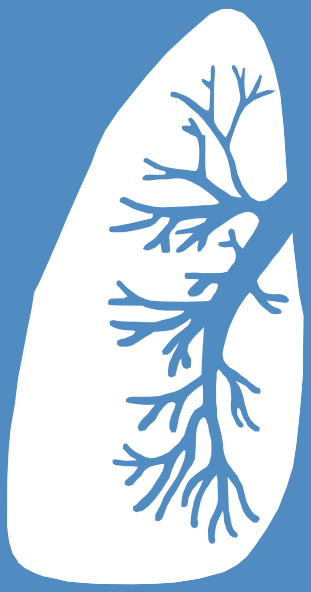
Part II

Pathogenesis of Respiratory Infections and Early Life Intervention



4

Chapter 4

Galacto-oligosaccharides Alleviate Lung Inflammation by Inhibiting NLRP3 Inflammasome Activation *in vivo* and *in vitro*

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Journal of Advanced Research (Revision submitted)

Abstract

Background and Purpose

The lack of effective anti-inflammatory therapies for pneumonia represents a challenge for identifying new alternatives. Non-digestible galacto-oligosaccharides (GOS) are attractive candidates due to their anti-inflammatory and immunomodulatory effects both locally and systemically. Therefore, the anti-inflammatory properties of GOS were investigated in lung infection models.

Experimental Approach

GOS were administrated orally to calves with naturally occurring lung infections during early life or used as pretreatments in cell cultures with primary bronchial epithelial cells (PBECs) exposed to *M. haemolytica*, lipopolysaccharides (LPS) or leukotoxin. The cell composition, cytokine/chemokine concentrations, and *M. haemolytica*-LPS IgG levels in broncho-alveolar lavage fluid (BALF) and blood were investigated, while the *M. haemolytica* positivity in BALF and bronchial mucosa was detected *in vivo*. Key markers of NLRP3 inflammasome activation were measured *in vivo* and *in vitro*.

Key Results

GOS reduced the *M. haemolytica* positivity and *M. haemolytica*-LPS IgG levels in calves with lung infections. Regulation of immune function and suppression of inflammatory response by GOS was related to the inhibition of NLRP3 inflammasome as observed in bronchial mucosal tissue of infected calves. The *M. haemolytica*-induced IL-1β production in PBECs was reduced by GOS, which was associated with NLRP3 inflammasome inhibition caused by the decreased ROS and ATP production. GOS inhibited leukotoxin-induced ATP production in PBECs. The LPS- and ATP-induced NLRP3 inflammasome activation in PBECs was suppressed by GOS.

Conclusion and Implications

GOS exert anti-inflammatory properties by inhibiting the NLRP3 inflammasome activation *in vitro* and *in vivo*, suggesting a potential role for GOS in the prevention of lung infections.

Keywords: Respiratory infections; *Mannheimia haemolytica*; IL-1β; Non-digestible oligosaccharides; Primary bronchial epithelial cells; Reactive oxygen species

Bullet point summary

What is already known

1. NLRP3 inflammasome is involved in lung inflammation and infection in humans and rodents.
2. GOS is one of the non-digestible oligosaccharides with prebiotic, anti-inflammatory and immunomodulatory effects.

What this study adds

1. GOS regulate immune function and suppress local and systemic inflammation in lung infections.
2. GOS inhibit NLRP3 inflammasome activation caused by *M. haemolytica*, LPS or ATP.

Clinical significance

1. GOS can be a promising future strategy for preventing lung infections and subsequent inflammation.

Introduction

Lung infection is the single biggest cause of pediatric death worldwide [1] and one of the most common causes of morbidity and mortality in calves [2]. The calf is considered to be an excellent animal model of human (childhood) lung infections: the prevalence of pneumonia is extremely high, involving the most important airborne and close contact transmission [3]. *Mannheimia haemolytica* is one of the principal Gram-negative bacteria in calves that causes lung infections characterized by a decline in innate immune function, dysfunction of airway epithelium and a large influx of inflammatory mediators (e.g., IL-1β, TNF-α) into the airways [2, 4]. The severity of *M. haemolytica* infection is likely determined by its virulence factors, such as lipopolysaccharides (LPS), which can activate Toll-like receptor 4 (TLR4) [2, 5]. Despite extensive research, antibiotics remain the mainstay for the treatment of lung infections.

Recently, it was found that NLR family pyrin domain containing 3 (NLRP3) inflammasome is strongly involved in lung inflammation and infection in humans and rodents [1]. The NLRP3 inflammasome is a unique inflammasome, whose activation is a two-step process induced by various microbial molecules (e.g., LPS) or danger signals (e.g., ROS, ATP) [1]. Firstly, a priming event induced by TLR/NF-κB signaling increases the expression of NLRP3 and pro-IL-1β. The second signal triggers inflammasome multimerization and IL-1β maturation. The NLRP3 inflammasome can be activated by multiple respiratory pathogens, including *S. aureus* [6], *S. pneumoniae* [7] and *K. pneumoniae* [8], which indicates the NLRP3 inflammasome could be a potential therapeutic and preventive target for lung infections [1].

Non-digestible oligosaccharides (NDOs) have the potential to prevent respiratory diseases due to their prebiotic, anti-inflammatory and immunomodulatory effects [9, 10]. There are some indications that NDOs could be effective against respiratory infections [11-14]. Oral acidic oligosaccharides derived from pectin increased bacterial clearance in mice with a *P. aeruginosa*-induced lung infection [11]. NDO mixtures containing galacto-/fructo-oligosaccharides (GOS/FOS) prevented, particularly, respiratory infections during the first 6 months of age [12] and reduced the frequency of respiratory infections, and antibiotic prescriptions in the first two years of life [13]. In addition, GOS supplementation decreased the duration and symptoms of cold or flu among university students [14]. More mechanism-related research is required to investigate the possibility of using GOS as a strategy to prevent respiratory infections.

Here, we investigated whether GOS would alleviate airway inflammation in

calves with lung infections and tried to unravel the mechanism by using *in vitro* systems with calf primary bronchial epithelial cells (PBECs) and human lung epithelial cells stimulated with *M. haemolytica*/LPS/ATP. For the first time, the importance of NLRP3 inflammasome activation in a bovine lung infection has been demonstrated, whereas GOS mitigated the infection-induced inflammatory response, which might be explained by the inhibition of the NLRP3 inflammasome activation. GOS supplementation with or without the combination of standard drugs might be a promising future strategy to combat respiratory infections.

Results

Remission of lung infections and inhibition of inflammation by GOS

All calves were naturally exposed to respiratory pathogens in the environment. GOS were orally (twice/day) administrated to calves from experimental week 1 till 8 to manipulate early-life conditions, thereafter the animals were followed from week 9 till 27 without GOS administration (**Figure 1A** and **B**). At week 27, all calves received lung scores after slaughter. Oral 2% GOS tended to show lower proportion of moderate/severe lung lesions (31%, p=0.09) compared with the control group (44%, **Figure 1C**).

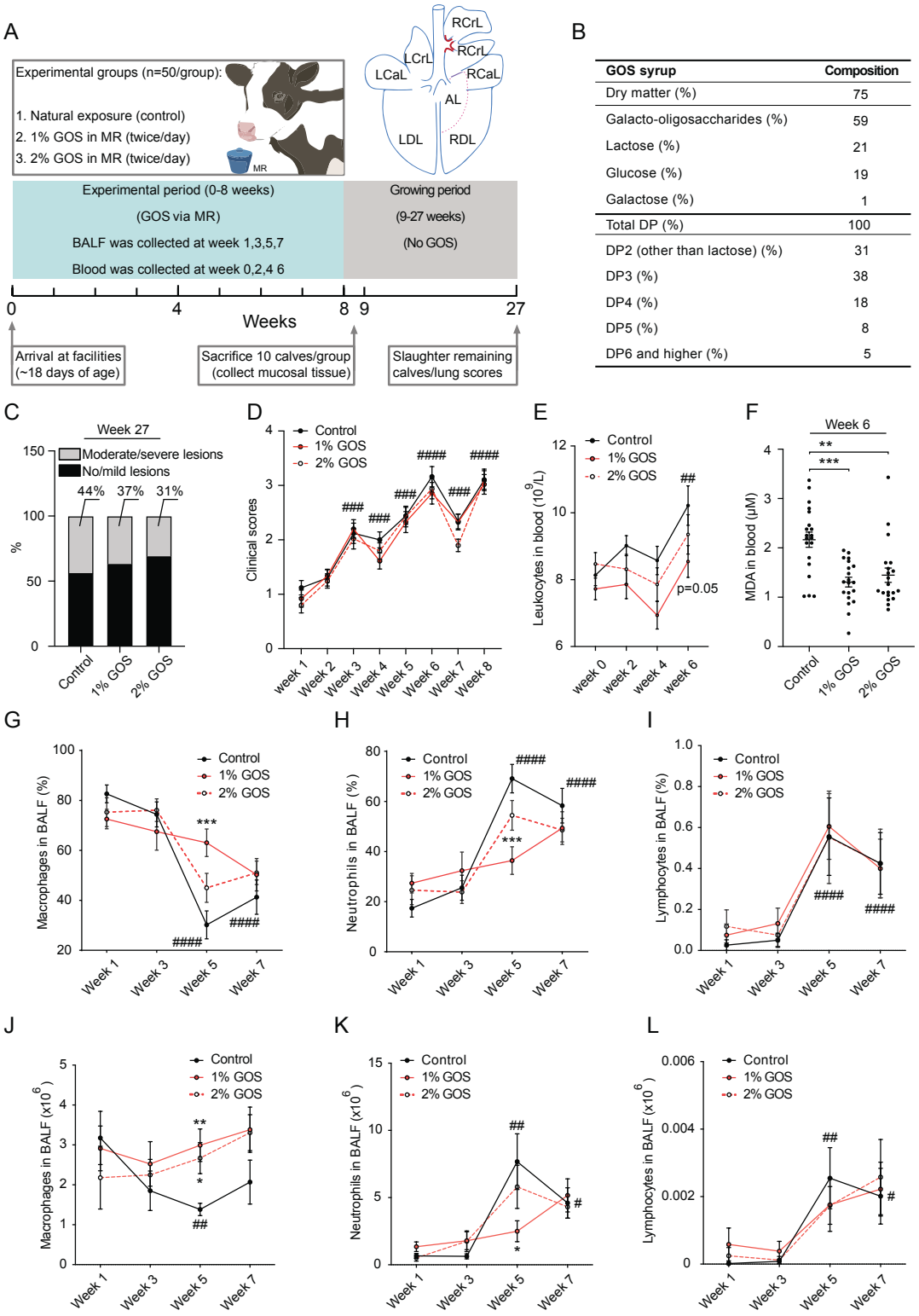
To study the extent of lung infection in calves upon GOS administration, the blood and broncho-alveolar lavage fluid (BALF) was examined at week 0, 2, 4, 6 and week 1, 3, 5, 7, respectively (**Figure 1A**). Increased leukocytes (increased by 26%) in blood and total cells (mainly pulmonary leukocytes; increased by 92%) in BALF of control calves were observed at week 6 and 5, respectively (**Figure 1E** and **S1A**). Decreased percentage and number of macrophages (number decreased by 56%) and increased percentage and number of neutrophils (number increased by 11-fold) and lymphocytes (number increased by 138-fold) were observed from week 5 in control calves (**Figure 1G-L**). Furthermore, the clinical scores increased significantly over time with the peak at week 6 (**Figure 1D**). These findings indicate that lung infections were present in these calves from week 5.

Control calves displayed a significant increase in blood leukocyte concentrations, which increased by 26% at week 6 compared with week 0, while 1% GOS tended to (p=0.05) decrease the concentrations of leukocytes at week 6 (**Figure 1E**). Furthermore, dietary GOS revealed a significant reduction in malondialdehyde (MDA) levels (a biomarker for oxidative stress) in blood

compared with control calves at week 6 (**Figure 1F**). In addition, GOS showed no significant effects on clinical scores, a scoring system for rectal temperature, coughing, nasal discharge and behavior, during the experimental period (**Figure 1D**).

Dietary GOS significantly increased the percentage and number of macrophages and decreased the percentage and number of neutrophils in BALF at week 5 (**Figure 1G**, **H**, **J**, and **K**), compared with the control animals. Interestingly, GOS had no effects on the lymphocytes (**Figure 1I** and **L**). In addition, none of the GOS treatments did affect the total cell numbers in BALF (**Figure S1A**). Overall, GOS at 1% restored the imbalance of the ratio of macrophages to neutrophils in BALF caused by lung infections at week 5.

The inflammatory response in the lungs was investigated by measuring proinflammatory cytokines/chemokines in BALF. GOS significantly reduced the concentrations of IL-8, TNF-α, IL-6, and IL-1β in BALF at week 5 (**Figure 2A-D**). The same cytokines/chemokines were measured in blood to investigate the effect of GOS on systemic inflammation caused by lung infections at week 4 and 6 (one week prior to or one week after week 5). In control group, the IL-8 and TNF-α levels in blood were significantly increased at week 6 compared with week 4, while IL-6 and IL-1β concentrations were slightly reduced (**Figure 2E-H**). GOS significantly reduced the IL-8, IL-6 and IL-1β levels in the blood at both week 4 and 6 (**Figure 2E-H**).



GOS syrup	Composition
Dry matter (%)	75
Galacto-oligosaccharides (%)	59
Lactose (%)	21
Glucose (%)	19
Galactose (%)	1
Total DP (%)	100
DP2 (other than lactose) (%)	31
DP3 (%)	38
DP4 (%)	18
DP5 (%)	8
DP6 and higher (%)	5

Figure 1. Effect of GOS on lung and clinical scores as well as on cell composition in BALF and blood. (**A**) Timeline and design of the experiment. Calves were naturally exposed to respiratory pathogens in the environment and treated twice per day with or without GOS orally for 8 weeks during early life followed by 19 weeks (week 9-27) without

GOS administration. BALF and blood were collected at week 1, 3, 5, 7 and week 0, 2, 4, 6, respectively. Bronchial mucosal tissue (red area around the right cranial lobe) was collected at week 8. Lung scores were assessed at week 27. (**B**) GOS syrup composition. (**C**) Proportion of different lung lesions in calves was calculated based on the lung scores at week 27 (n=107, 34-37 calves/group). (**D**) Clinical scores were evaluated over time (n=150, 50 calves/group). (**E**) Concentration of leukocytes in blood was measured at week 0 (n=150, 50 calves/group) and week 2, 4, and 6 (n=60, 20 calves/group). (**F**) MDA concentrations in blood were assessed at week 6 (n=60, 20 calves/group). Each dot represents one calf. (**G**-**L**) Percentage and number of macrophages, neutrophils, and lymphocytes in BALF were measured at week 1, 3, 5 and 7 (n=60, 20 calves/group). *P*=0.05, **P*<0.05, ***P*<0.01, ****P*<0.001, (GOS treatments vs control group); #*P*<0.05, ##*P*<0.01, ###*P*<0.001, ####*P*<0.0001 (control week 3-8 vs week 0 or 1). Data are presented as means ± SEM. RCrL = right cranial lobe; RCaL = right cardiac lobe; RDL = right diaphragmatic lobe; AL = accessory lobe; LCrL = left cranial lobe; LCaL = left cardiac lobe; LDL = left diaphragmatic lobe; BALF = broncho-alveolar lavage fluid; DP = degree of polymerization; GOS = galacto-oligosaccharides; MR = milk replacer; MDA = malondialdehyde.

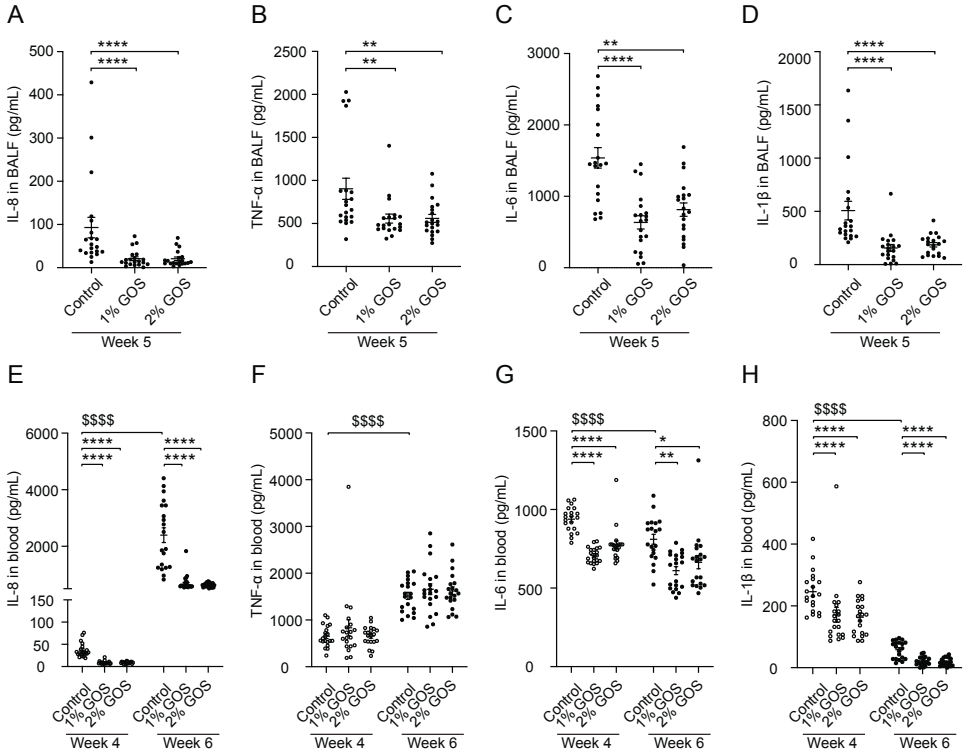


Figure 2. Effect of GOS on cytokine/chemokine measurements in BALF and blood. (**A**-**D**) Concentrations of IL-8, TNF-α, IL-6, and IL-1β in BALF were measured by ELISA at week 5 (n=60, 20 calves/group). (**E**-**H**) Levels of IL-8, TNF-α, IL-6, and IL-1β in blood were determined by ELISA at week 4 and 6 (n=60, 20 calves/group). **P*<0.05, ***P*<0.01, *****P*<0.0001 (GOS treatments vs control group); $$$$*P*<0.0001 (control week 6 vs week 4). Data are presented as means ± SEM. Each dot represents one calf. BALF = broncho-alveolar lavage fluid; GOS = galacto-oligosaccharides.

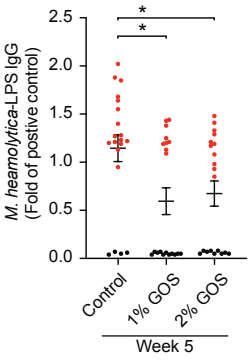
GOS reduce *M. haemolytica* positivity in calves

M. haemolytica is one of the main pathogens that contribute to the development of bovine lung infections. It releases LPS to produce proinflammatory cytokines/chemokines and promote the lung lesions through the stimulation of epithelial cells and leukocytes [15]. Here, the *M. haemolytica*-LPS IgG levels was detected in BALF and blood. Compared to week 1 (0%), the number of calves positive for *M. haemolytica* within the control group increased over time and reached 80% (16/20) at week 5 (**Table S1**). The effect of GOS on *M. haemolytica*-LPS IgG levels was investigated in BALF at week 5 and in blood at week 4 and 5 (the same timepoint for the measurements of cytokines/chemokines in BALF and/or blood). Interestingly, GOS reduced the *M. haemolytica*-LPS IgG levels in BALF and blood at week 5 and 6, respectively (**Figure 3A-B**).

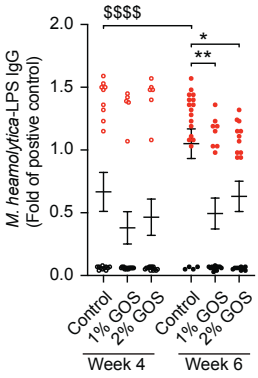
The detection of *M. haemolytica*-LPS IgG levels is an indirect method for identifying *M. haemolytica* in BALF. Hence, the positivity for *M. haemolytica* was detected by real-time PCR in BALF of all calves at week 5. **Figure 3C** showed that 80% (16/20) of control calves were positive for *M. haemolytica*. In line with the data from *M. haemolytica*-LPS IgG levels in BALF (**Table S1**), it indicated that *M. haemolytica* might be one of the pathogens involved in the lung infection of calves from week 5. Interestingly, administration of 1% and 2% GOS showed a reduction in the number of calves positive for *M. haemolytica* (45% and 55% of the calves were positive for *M. haemolytica* at week 5, respectively) (**Figure 3C**).

Infected lesions are most often observed in the right cranial lobe (RCrL) of bovine lungs in previous research [16] and current study as depicted in supplementary **Figure S1B**. The bronchial mucosal tissue nearby the RCrL was collected (**Figure 1A**) and the presence of *M. haemolytica* was identified in these tissues as well. 80% (8/10) of the calves showed that *M. haemolytica* was present in the bronchial mucosa. Although it is not statistically significant, 1% and 2% GOS reduced the number of calves positive for *M. haemolytica* (50% and 60% of the calves were positive for *M. haemolytica* in the bronchial mucosa at week 8, respectively) (**Figure 3C**).

A



B



C

Sample type	Real-time PCR method			*M. haemolytica* positivity		
				p-value (vs control)		
	Control	1%GOS	2%GOS	Control	1%GOS	2%GOS
BALF at week 5 (n=20 calves/group)	16	9	11	80%	45%	55%
				—	*p*=0.02	*p*=0.09
Bronchial mucosa (n=10 calves/group)	8	5	6	80%	50%	60%
				—	*p*=0.16	*p*=0.33

Figure 3. GOS reduce *M. haemolytica* positivity in calves. (**A**-**B**) *M. haemolytica*-LPS IgG levels was detected in BALF at week 5 (**A**) and in the blood at week 4 and 6 (**B**) by ELISA method (n=60, 20 calves/group). Compared to the positive control, fold changes were calculated. Black dots represent *M. haemolytica*-LPS IgG negative calves. Red dots represent *M. haemolytica*-LPS IgG positive calves. (**C**) Number and percentage of *M. haemolytica* positive calves according to the presence in BALF or bronchial mucosa by real-time PCR method. **P*<0.05, ***P*<0.01 (GOS treatments vs control group); $$$$*P*<0.0001 (control week 6 vs week 4). Data are presented as means ± SEM. BALF = broncho-alveolar lavage fluid; GOS = galacto-oligosaccharides; LPS = lipopolysaccharide.

Inhibition of the activation of NLPR3 inflammasome by GOS *in vivo*

The invasion of and damage to the bronchial mucosa by *M. haemolytica* may promote the production of inflammation (e.g., IL-1β release) and the formation of infection foci [1, 15]. The extent of NLRP3 inflammasome activation after GOS intervention was studied in the bronchial mucosal tissue. GOS significantly reduced the phosphorylation of NF-κB p65 and the expression of NLRP3, TLR4, and IL-1β (**Figure 4A**) in mucosal tissue. A decreased release of MDA and activation of caspase-1 were observed after GOS intervention (**Figure 4B** and **C**).

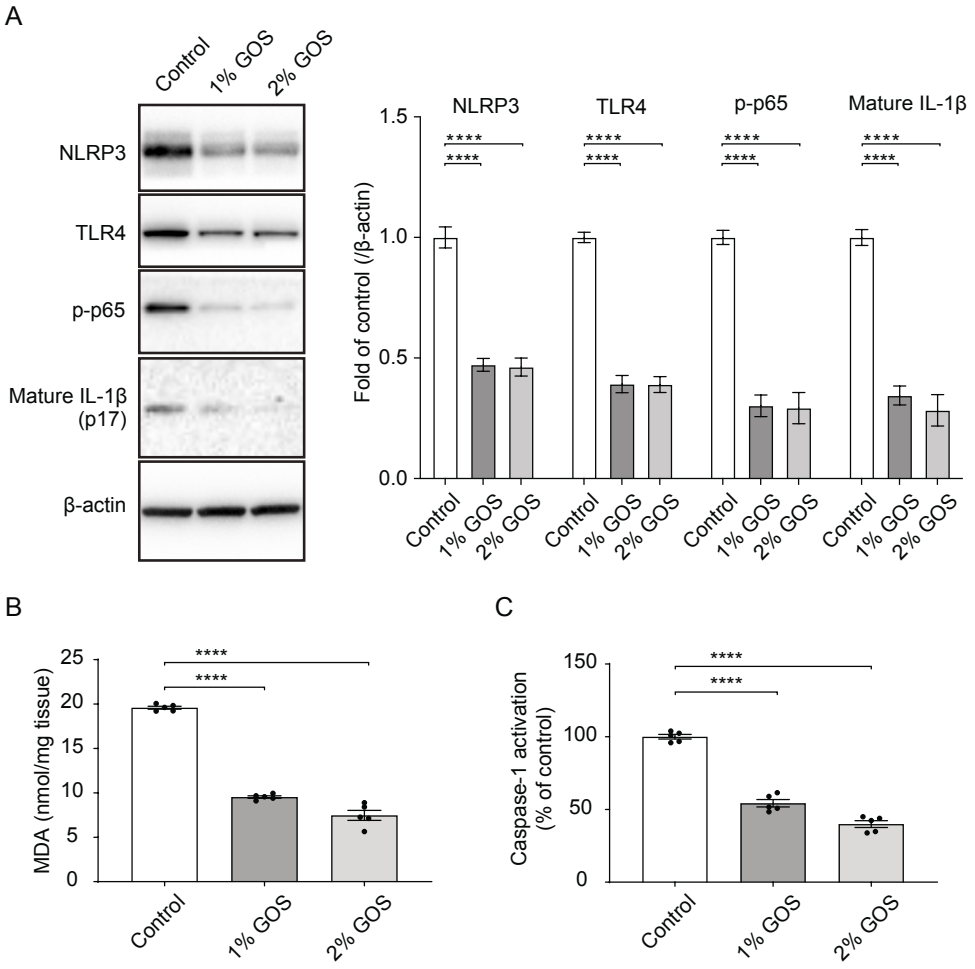


Figure 4. Inhibition of the activation of NLPR3 inflammasome by GOS *in vivo*. Calf bronchial mucosal tissue of control, 1% GOS and 2% GOS groups was collected at week 8. (**A**) Expression of NLRP3, TLR4 and IL-1β and phosphorylation of NF-κB p65 were determined by western blotting (n=15, 5 calves/group). (**B-C**) MDA release and caspase-1 activation were assessed in control, 1% GOS and 2% GOS groups (n=15, 5 calves/group). Each dot represents one calf. ****P<0.0001 (GOS treatments vs control group). Data are presented as means ± SEM. GOS = galacto-oligosaccharides; MDA = malondialdehyde; NLRP3 = NLR family pyrin domain containing 3; TLR4 = Toll-like receptor 4.

Inhibition of *M. haemolytica*-induced release of IL-1β and TNF-α by GOS in primary bronchial epithelial cells

Epithelial cells are one of the main cell types present in the bronchial mucosa and are the first line of defense against the invasion of pathogens [3]. To unravel the mechanism of GOS inhibiting *M. haemolytica*-induced inflammation, primary

epithelial cells near the RCrL of healthy lungs were collected and cultured. An *ex vivo* infection model with PBECs stimulated by *M. haemolytica,* an important pathogen possibly involved in lung infections in the present *in vivo* study, was developed [17].

In the present study, pretreatment with GOS concentration-dependently inhibited the *M. haemolytica*-induced release of IL-1β and TNF-α (**Figure 5A** and **B**), while it did not affect the cellular survival (MTT assay) and lactate dehydrogenase (LDH) release (**Figure 5C** and **D**).

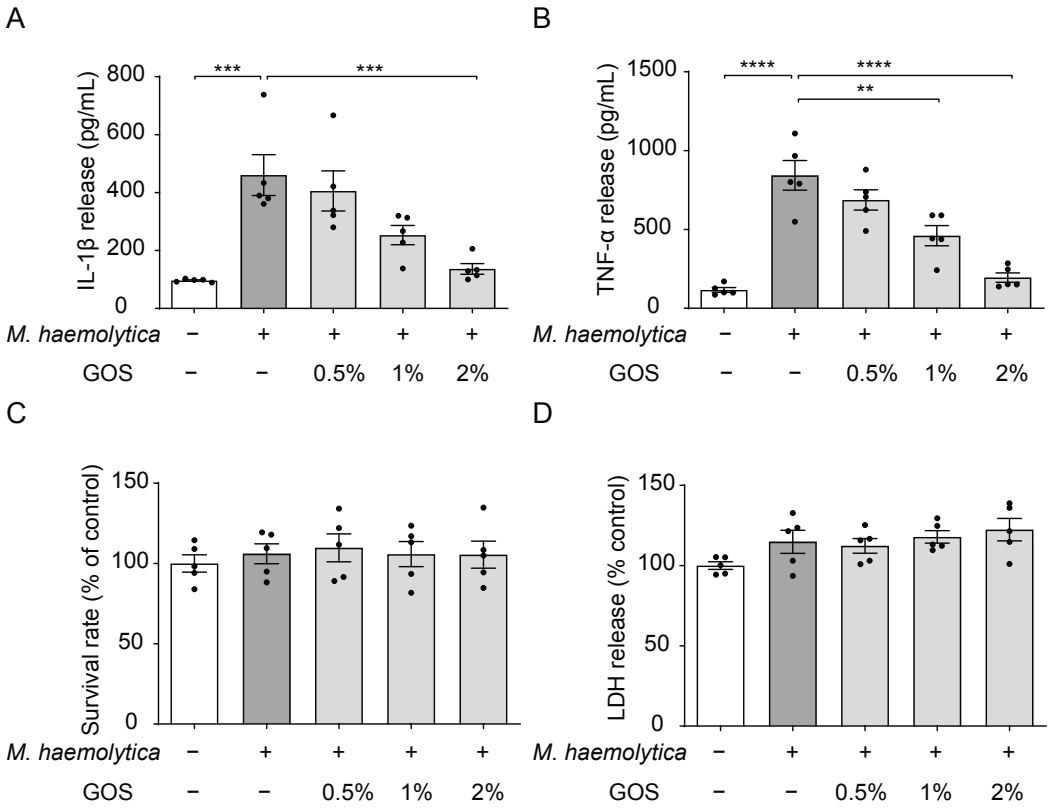


Figure 5. Inhibition of *M. haemolytica*-induced release of IL-1β and TNF-α by GOS in primary bronchial epithelial cells. PBECs were incubated with *M. haemolytica* (1 x 10^5 CFU/mL) for 24h with or without 24h pretreatment with GOS. (**A-B**) IL-1β and TNF-α release were measured in the supernatants of PBECs. (**C-D**) Survival rates were determined by the percentage of MTT levels in PBECs and LDH release was assessed in the supernatants. ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves). GOS = galacto-oligosaccharides; LDH = lactate dehydrogenase; MTT = thiazolyl blue tetrazolium bromide; PBECs = primary bronchial epithelial cells.

Inhibition of *M. haemolytica*-induced activation of NLPR3 inflammasome by GOS in primary bronchial epithelial cells

To investigate the activation of NLPR3 inflammasome *in vitro*, IL-1β and NLRP3 expression, mitochondrial function and caspase-1 activation were examined. Western blotting of cell lysates and ELISA data showed that 24h pretreatment with GOS significantly inhibited the *M. haemolytica*-induced expression and release of mature IL-1β, respectively, which was also observed after preincubation with NLRP3 inflammasome inhibitor MCC950 (**Figure 6A** and **B**). Furthermore, pretreatment with GOS inhibited the *M. haemolytica*-induced activation of caspase-1 and production of ATP, ROS, and MDA (**Figure 6C-F**). Remarkably, by pretreating PBECs with ROS inhibitors, acetylcysteine (NAC) significantly decreased *M. haemolytica*-induced IL-1β release (**Figure S2**).

In addition, GOS significantly inhibited the *M. haemolytica*-induced expression of NLRP3 as measured by western blotting and immunofluorescence staining, which was comparable to the inhibitory effect of MCC950 (**Figure 6G** and **H**). Moreover, GOS also significantly decreased the expression of TLR4, and the phosphorylation of p38, ERK1/2, JNK1/2 MAPK and NF-κB p65 (**Figure 6I**).

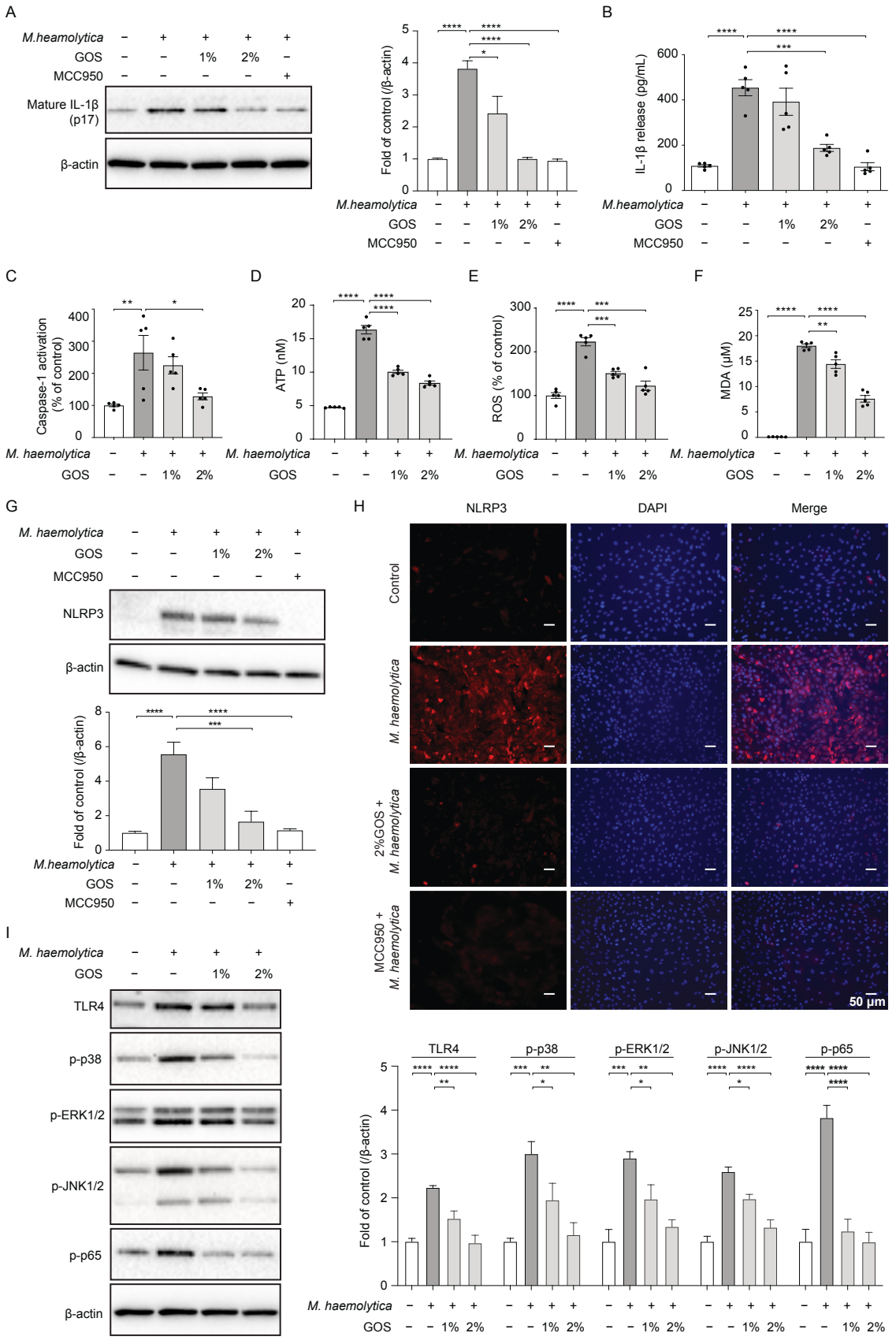

A
M.heamolytica
GOS
MCC950
Mature IL-1β (p17)
β-actin
Fold of control (/β-actin)
B
IL-1β release (pg/mL)
C
Caspase-1 activation (% of control)
D
ATP (nM)
E
ROS (% of control)
F
MDA (μM)
M. haemolytica
G
NLRP3
H
NLRP3
DAPI
Merge
Control
M. haemolytica
2%GOS + M. haemolytica
MCC950 + M. haemolytica
50 μm
I
TLR4
p-p38
p-ERK1/2
p-JNK1/2
p-p65
β-actin

Figure 6. Inhibition of *M. haemolytica*-induced activation of NLPR3 inflammasome by GOS in primary bronchial epithelial cells. PBECs were incubated with *M. haemolytica* (1 x 10^5 CFU/mL) for 24h with or without GOS (24h) or MCC950 (6h) pretreatment. (**A-B**) Expression of mature IL-1β in cell lysates and release of IL-1β in the supernatants were examined and data were shown as a fold of control or absolute amount of IL-1β. (**C-F**) Caspase-1 activation and ATP, ROS, and MDA production in PBECs were assessed and data were shown as a percentage of control or absolute amount. (**G**) Expression of NLRP3 was determined by immunoblot and results were shown as a fold of control. (**H**) PBECs were stained for NLRP3 (red), followed by counterstaining with DAPI (blue). (**I**) Expression of TLR4 and phosphorylation of p38, ERK1/2, JNK1/2 MAPK and NF-κB p65 were determined by immunoblot and results were shown as a fold of control. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves). ATP = adenosine triphosphate; GOS = galacto-oligosaccharides; MDA = malondialdehyde; NLRP3 = NLR family pyrin domain containing 3; PBECs = primary bronchial epithelial cells; ROS = reactive oxygen species; TLR4 = Toll-like receptor 4.

Inhibition of LPS-induced NLPR3 inflammasome activation in both bovine primary bronchial epithelial cells and human lung epithelial cells by GOS

M. haemolytica colonize and invade the bronchial mucosa with the help of released LPS [15]. Host cells prime the formation of NLRP3 inflammasome through recognizing LPS released by pathogens [1]. Here, GOS on LPS-triggered activation of NLRP3 inflammasome was investigated.

In the present study, LPS exposure resulted in a significant release in IL-1β and activation in caspase-1, which was facilitated by the production of ROS and MDA (**Figure 7A-D**) in PBECs. In contrast, pretreatment with GOS significantly inhibited the LPS-induced release of IL-1β and activation of caspase-1, as well as the production of ROS and MDA (**Figure 7A-D**), while GOS alone did not affect the IL-1β release and ROS production. The ROS inhibitor (NAC) was also able to significantly inhibit the LPS-induced IL-1β release (**Figure S2**). Furthermore, GOS was also effective in inhibiting the ROS production in rotenone stimulated PBECs (positive control) (**Figure S3**).

In addition, the increased phosphorylation pattern of NF-κB p65 and expression pattern of NLRP3 showed priming of NLRP3 inflammasome in LPS-treated PBECs (**Figure 7E**). Pretreatment with GOS significantly decreased the NF-κB p65 phosphorylation and the NLRP3 expression, while GOS alone did not affect these expression patterns (**Figure 7E**). Moreover, LPS with or without GOS pretreatment and GOS alone did not affect the cellular survival and LDH release in PBECs (**Figure S4**).

To verify the key findings obtained with bovine cells, human lung epithelial cells (A549) were preincubated with GOS and stimulated with LPS for 24h, as described for PBECs. GOS preincubation decreased NLRP3 inflammasome activation induced by LPS as monitored by analyzing IL-1β release, ROS and MDA production and caspase-1 activation in human A549 cells (**Figure 7F-I**).

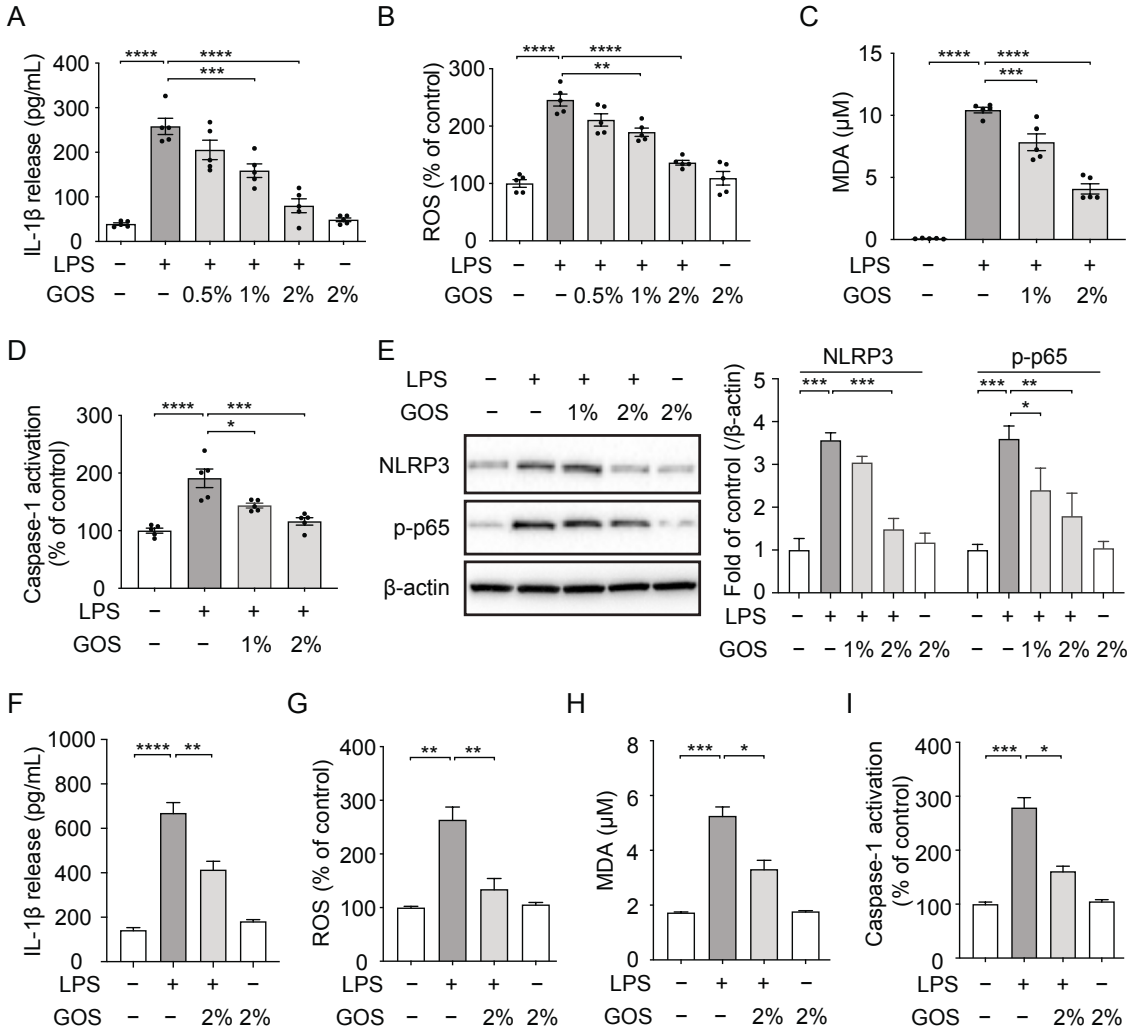


Figure 7. Inhibition of LPS-induced NLPR3 inflammasome activation in both bovine primary bronchial epithelial cells and human lung epithelial cells by GOS. PBECs or A549 cells were treated with LPS for 24h with or without 24h pretreatment with GOS. (**A**) The IL-1β release was measured in the supernatants of PBECs. (**B**-**C**) The mitochondrial ROS and MDA production were assessed in PBECs. (**D**) The caspase-1 activation was examined in PBECs. (**E**) Expression of NLRP3 and phosphorylation of NF-κB p65 were determined in PBECs and results were shown as a fold of control. (**F**) The IL-1β release was measured in the supernatants of A549 cells. (**G**-**H**) The mitochondrial ROS and MDA production were assessed in A549 cells. (**I**) The caspase-1 activation was examined in A549 cells. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves or cell generations). ATP = adenosine triphosphate; GOS = galacto-oligosaccharides; LPS = lipopolysaccharide; MDA = malondialdehyde; NLRP3 = NLR family pyrin domain containing 3; PBECs = primary bronchial epithelial cells; ROS = reactive oxygen species.

Inhibition of leukotoxin A-induced production of ATP by GOS in primary bronchial epithelial cells

Similar to LPS, leukotoxin is also responsible for lung inflammation caused by *M. haemolytica* [15]. Here, IL-1β and ATP production in PBECs after exposure to leukotoxin A (secreted by *M. haemolytica*) with or without 24h GOS pretreatment was evaluated. Despite leukotoxin A-exposed PBECs did not show a significant increase in IL-1β release within 24h stimulation, a significant increase in ATP production was observed after 6h (**Figure 8A** and **C**). No cytotoxic effects of leukotoxin A on PBECs were observed till 12h incubation (**Figure 8B**). The leukotoxin A-induced ATP production (after 12h) was inhibited by preincubation with GOS (**Figure 8D**). The inhibition of ATP production by GOS was also observed in *M. haemolytica*-treated PBECs (**Figure 5D**), while GOS treatment alone did not affect ATP production (**Figure S5**).

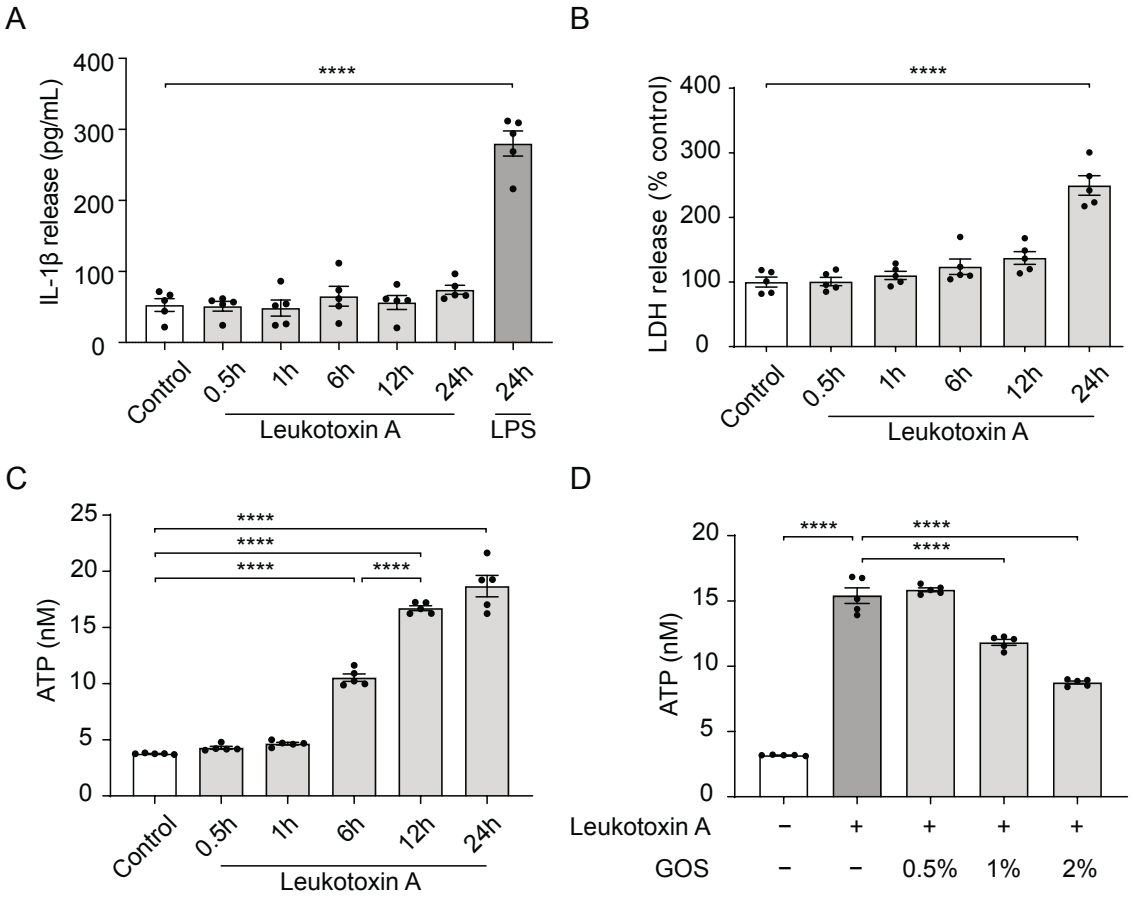


Figure 8. Inhibition of leukotoxin A-induced production of ATP by GOS in primary bronchial epithelial cells. (**A**) PBECs were incubated with leukotoxin A for different timepoints (0.5, 1, 6, 12 and 24h) or LPS for 24h, and the IL-1β release was measured

in the supernatants. (**B**-**C**) PBECs were incubated with or without leukotoxin A for different timepoints (0.5, 1, 6, 12 and 24h) and the LDH release and ATP production were examined. (**D**) PBECs were incubated with leukotoxin A for 12h with or without the 24h pretreatment with GOS and the ATP production was analyzed. ****P<0.0001. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves). ATP = adenosine triphosphate; GOS = galacto-oligosaccharides; LPS = lipopolysaccharide; LDH = lactate dehydrogenase; PBECs = primary bronchial epithelial cells.

Inhibition of ATP-induced NLPR3 inflammasome activation in both bovine primary bronchial epithelial cells and human lung epithelial cells by GOS

Although leukotoxin A failed to promote IL-1β release within 24h in PBECs, it induced ATP production within 6h. ATP, as an endogenous danger signal, has been reported to trigger the activation of NLRP3 inflammasome via the induction of K^+ efflux (signal 2) [1]. Here, the IL-1β release and caspase-1 activation in PBECs increased after LPS+ATP (6h+0.5h) stimulation, although LPS (6h) or ATP (0.5h) alone could not significantly increase IL-1β release (**Figure 9A** and **B**). Therefore, the effect of GOS on LPS+ATP-induced activation of NLRP3 inflammasome was investigated.

Interestingly, GOS displayed a significant decrease in IL-1β release, caspase-1 activation, and ROS and MDA production in PBECs exposed to LPS+ATP (**Figure 9A-D**). In addition, the ROS inhibitor, NAC, significantly inhibited LPS+ATP-induced IL-1β release (**Figure S2**).

To verify the findings obtained with bovine cells, human A549 cells were preincubated with GOS for 24h and stimulated with LPS+ATP, as described for PBECs. GOS preincubation decreased NLRP3 inflammasome activation induced by LPS+ATP as monitored by analyzing IL-1β release, caspase-1 activation and ROS and MDA production in human A549 cells (**Figure 9E-H**).

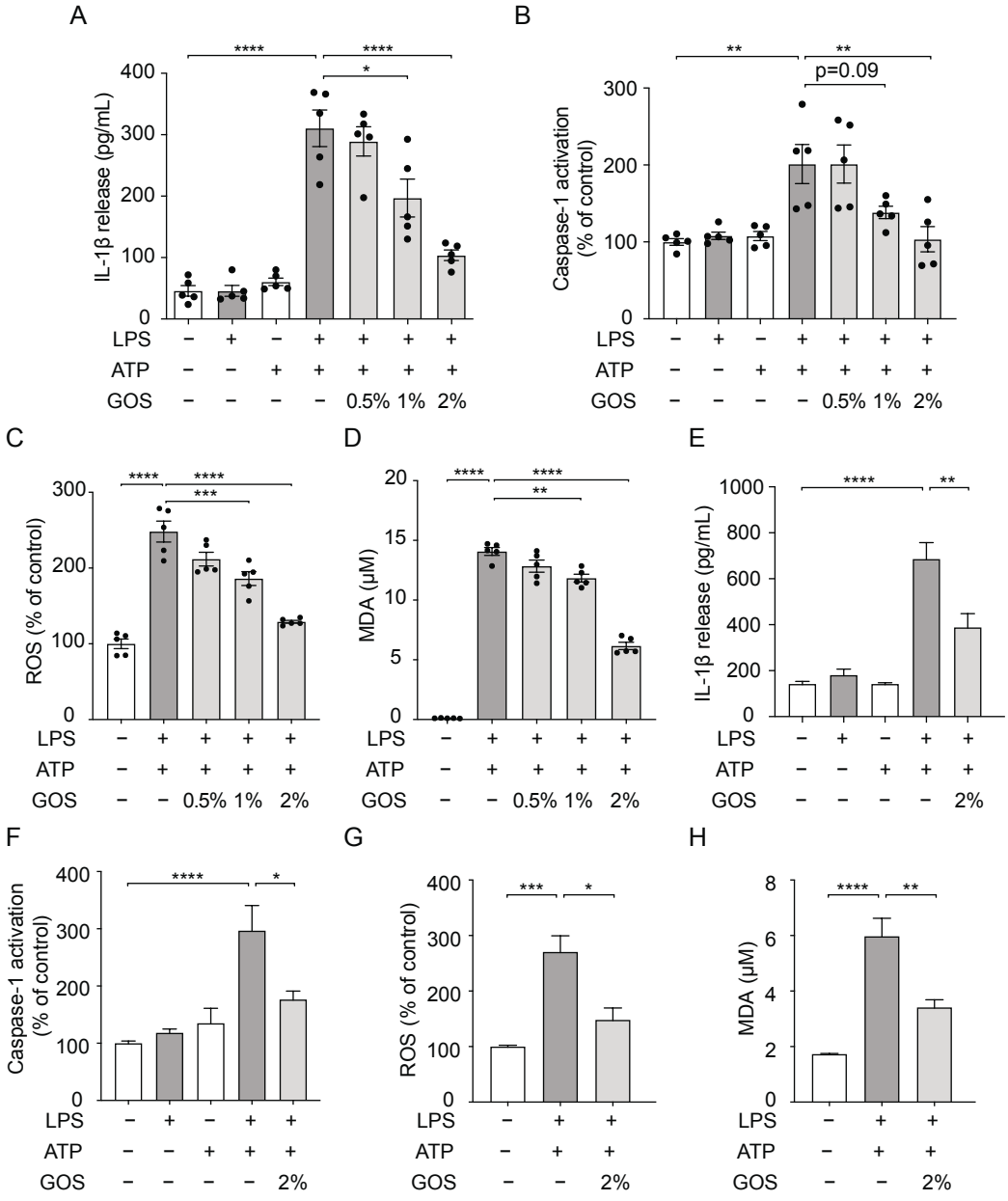


Figure 9. Inhibition of ATP-induced NLPR3 inflammasome activation in both bovine primary bronchial epithelial cells and human lung epithelial cells by GOS. PBECs or A549 cells were treated with or without GOS pretreatment (24h) prior to the stimulation by LPS (6h) or ATP (0.5h) or LPS+ATP (6h+0.5h). (**A**) The IL-1β release was measured in the supernatants of PBECs. (**B**) The caspase-1 activation was examined in PBECs. (**C**-**D**) The mitochondrial ROS and MDA production were assessed in PBECs. (**E**) The IL-1β release was measured in the supernatants of A549 cells. Panel E has the same control group as Figure 7F. (**F**) The caspase-1 activation was examined in A549 cells. Panel F has the same control group as Figure 7I. (**G**-**H**) The mitochondrial ROS and MDA production were assessed in A549 cells. Panel G and H have the same control group as Figure 7G and H, respectively. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of five

independent experiments (n=5 donor calves or cell generations). ATP = adenosine triphosphate; GOS = galacto-oligosaccharides; LPS = lipopolysaccharide; MDA = malondialdehyde; PBECs = primary bronchial epithelial cells; ROS = reactive oxygen species.

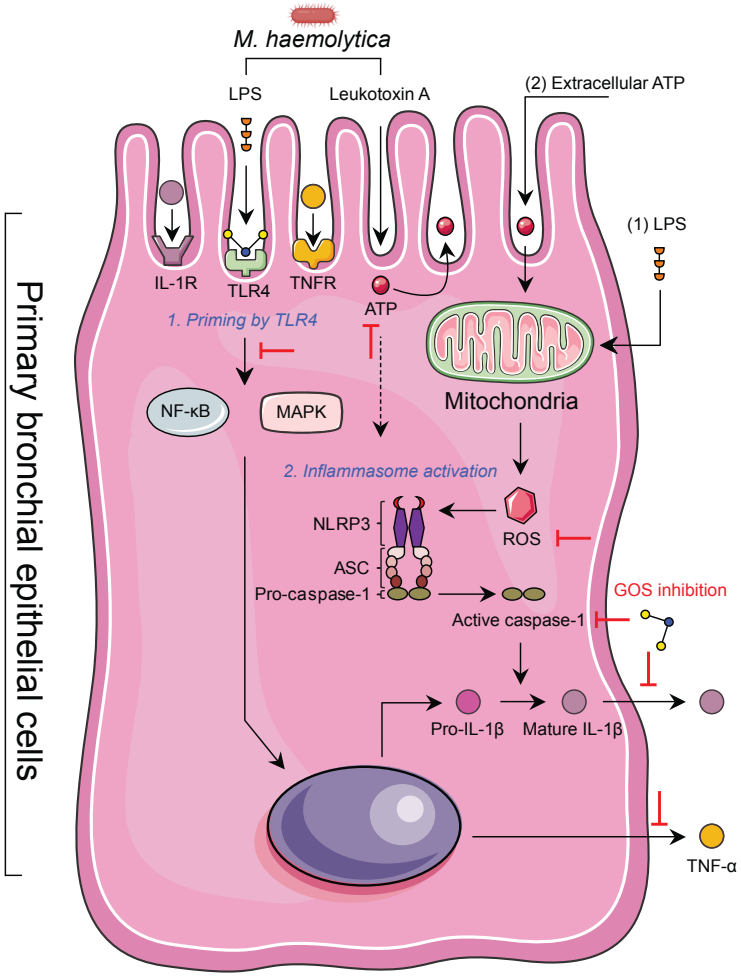


Figure 10. Pathogenesis of *M. haemolytica*-induced inflammation and potential treatment by GOS supplementation leading to inhibition of NLRP3 inflammasome. Infection of bronchial epithelial cells by respiratory pathogens (e.g., *M. haemolytica*) induces NLRP3 inflammasome activation that cleaves pro-caspase-1 into caspase-1, which in turn cleaves pro-IL-1β into mature IL-1β, causing systemic inflammation. *M. haemolytica* and the released virulence factors (e.g., LPS and leukotoxin A) might activate NLRP3 inflammasome in two different ways. (1) LPS directly activate NLRP3 inflammasome by being recognized by TLR4 and inducing mitochondrial ROS production and/or (2) NLRP3 inflammasome activation is a two-step process, with LPS as the initial priming signal mediated by TLR4 recognition, and extracellular ATP as the subsequent activation signal. Leukotoxin A might play a role in NLRP3 inflammasome activation by

increasing ATP production. GOS supplementation can inhibit the activation of NLRP3 inflammasome by reducing the release of TNF-α, the expression of TLR4, and the production of ATP and mitochondrial ROS, thereby suppressing/preventing lung inflammation (IL-1β release). ASC = adaptor protein apoptosis-associated speck-like containing a caspase recruitment domain; ATP = adenosine triphosphate; GOS = galacto-oligosaccharides; IL-1 = interleukin-1; LPS = lipopolysaccharides; MAPK = mitogen-activated protein kinase; NF-κB = nuclear factor kappa B; NLRP3 = NLR family pyrin domain containing 3; ROS = reactive oxygen species; TNF = tumor necrosis factor; TLR4 = Toll-like receptor 4.

Discussion

Altogether, this study indicated that dietary GOS can reduce airway and systemic inflammation by restoring the immune imbalance as well as decreasing the NLRP3 inflammasome activation at least regarding the early stages after lung infection was induced. Direct anti-inflammatory and anti-oxidative effects of GOS on lung cells are possibly involved as well.

Single-pathogen challenge model experimentally inoculates a large number of pathogens (e.g., *M. haemolytica*) in the lower respiratory tract [18], however, ignore the complexity of infection, involving the invasion of opportunistic bacteria caused by stress, weakened immunity, contact transmission, etc. [15]. However, in natural exposure or inoculated infections, clinically healthy or asymptomatic individuals might be present [18, 19], which is like the pattern of human respiratory infections. This could be the reason why the effect of GOS on BALF composition and clinical scores might still be underestimated. In addition, the insensitivity of clinical scores to the diagnosis of (subclinical) lung infections may also lead to contrasting results as compared to the measurements of cell composition and cytokine/chemokine levels in BALF/blood [20]. Furthermore, the immunomodulatory effects of GOS occurred during week 5 and seemed to disappear during week 7, which might be related to (1) the group antibiotic treatments for all calves based on clinical scores at week 6 and/or (2) the innate immune system activation (increased BALF neutrophils and blood leukocytes) during week 5/6, which contributes to partly eliminating/phagocytosing the pathogens in the lungs.

Interestingly, up to 80% of calves are positive for *M. haemolytica* in BALF and bronchial mucosa at week 5 and 8, respectively. It has recently been reported that outbreaks of *M. haemolytica* infections increased in Dutch calves [21]. Although the presence of other opportunistic pathogens, such as *Pasteurella*

multocida was not investigated in our study, substantial evidence is provided to indicate that *M. haemolytica* might be one of the pathogens involved in the present lung infections. LPS plays a critical role in the pathogenesis of *M. haemolytica*-induced pneumonia, especially in the promotion of airway inflammation [15]. The presence of *M. haemolytica* released LPS in BALF and blood might be the cause of airway and systemic inflammation observed in lung infections, especially related to the IL-1β release caused by the NLRP3 inflammasome activation.

Oxidative stress-related NLRP3 inflammasome may play a central role in several inflammatory conditions in cows, while antioxidant supplementation during the peripartum period is beneficial for cow's health [22]. Others showed that an oxidative burst is caused during the adhesion of *M. haemolytica* to bovine neutrophils [23], and increased oxidative stress, as measured by MDA levels, was detected in the serum of goats infected with *M. haemolytica* [24]. In the present study, increased lipid peroxidation was detected in the blood and bronchial mucosa in control calves, which could be due to the invasion of *M. haemolytica* and released virulence factors (e.g., LPS, leukotoxin) [4, 15].

TLR4 on the airway epithelial cells mainly senses bacterial LPS and induces recognition to many Gram-negative pathogens [5]. In our study, *M. haemolytica* may release LPS to activate the "TLR4/NF-κB" pathway in PBECs, indicating the initiation of NLRP3 inflammasome activation (signal 1). A study in mice showed that inhalation of LPS induces an increase in TLR4 expression in bronchial epithelium and macrophages within 24h [25]. In addition, the activation of NLRP3/ASC inflammasome by the respiratory syncytial virus in human lung epithelial cells is primed by TLR4 [26], supporting our findings that *M. haemolytica* active NLRP3 inflammasome via the recognition of released LPS by TLR4 in PBECs.

Notably, the generation of ROS/ATP caused by *M. haemolytica* or its virulence factors (LPS and leukotoxin) results in rapid activation of NLRP3 inflammasome in PBECs (signal 2). Studies in human lung epithelial cells showed that LPS exposure for 24h resulted in strongly elevated ROS levels accompanied by mitochondrial dysfunction [27], which is in line with our data. It is possible that (long-term) LPS-induced ROS triggers the activation of NLRP3 inflammasome, which is also observed in our previous study where LPS and cigarette smoke activate NLRP3 inflammasome and induce IL-1β release in human bronchial epithelial cells within 16h [28]. In line with these findings, we observed that NAC can reverse the IL-1β release caused by LPS exposure. Furthermore, other studies showed that exposure to extracellular ATP resulted in increased ROS

production in human intestinal or gingival epithelial cells, which may be related to the activation of P2X7 receptors contributing to autophagy [29] or microbial infection [30]. Although in our study leukotoxin A failed to induce IL-1β release within 24h, it did cause rapid ATP release after 6h exposure to PBECs, which may trigger the (short-term) LPS-primed NLRP3 inflammasome activation.

NLRP3 inflammasome might be activated by ATP-dependent and ATP-independent mechanisms in PBECs stimulated with *M. haemolytica* (**Figure 10**). 1) long-term LPS exposure (24h) activates the "TLR4/NF-κB" pathway and increases mitochondrial ROS, leading to NLRP3 inflammasome activation accompanied by IL-1β release. 2) short-term LPS exposure (6h) needs an additional trigger, such as extracellular ATP, to initiate inflammasome activation.

Here, we reported for the first time that GOS have the potential to decrease IL-1β release via targeting/inhibiting NLRP3 inflammasome in an infection model.

NDOs have been shown to exert anti-oxidative effects. An *in vivo* study showed a reduction in renal injury, which is probably the result of a decrease in blood oxidative stress induced by GOS-mediated microbiota changes [31]. Although mechanisms remained unclear, *in vitro* studies showed that *Lactobacillus spp.* and *Bifidobacterium spp.* fermented by several NDOs cause oxygen-free radical elimination and lipid peroxidation inhibition [10, 32]. Although not investigated in the current study, it may be possible that changes in gut microbiota induced by dietary GOS contribute to the decreased NLRP3 inflammasome activation and corresponding factors [33].

In vivo data indicated that 1% GOS supplementation seems to be better than 2% GOS, especially in the measurements of immune parameters (the proportion and number of leukocytes). This might be due to the increase in stool frequency and changes in stool consistency by excessive consumption and fermentation of NDOs may interfere with the absorption of nutrients [34], especially, in infected calves.

Increasing evidence showed oligosaccharides are absorbed into the systemic circulation after oral administration [35, 36]. After oral ingestion of breast milk, about 1% of human milk oligosaccharides (HMOs) were absorbed in the blood circulation of infants [37]. Eiwegger *et al.* showed a 14% uptake of GOS across the intestinal epithelial layer *in vitro* [38]. Our previous study reported that GOS was detected in blood serum and urine of piglets after feeding 0.8% GOS once per day [37]. These studies suggest that GOS might reach the lungs (bronchus) through systemic circulation, resulting in direct inhibition of NLRP3 inflammasome and inflammation.

Interestingly, in addition to inhibiting the adhesion of pathogens to host epithelial cells, GOS have been found to act as TLR4 ligands to regulate host immune function, which could affect phosphorylation of NF-κB and cytokine production [10, 39]. Comparable to LPS, GOS might competitively bind to TLR4 of the bronchial epithelium, attenuating the priming of NLRP3 inflammasome (**Figure 10**). HMOs have been reported to inhibit the release of IL-8 and the phosphorylation of ERK and NF-κB caused by *E. coli* invasion of intestinal epithelial cells, which may be due to the reduction of CD4 binding to TLR4 [40]. Another *in vitro* study pointed out that chitosan-oligosaccharides (COS) can inhibit the activation of MAPK and NF-κB and the production of IL-1β and NO in LPS-treated RAW 264.7 cells, possibly because COS suppress the binding of LPS to the TLR4/MD-2 receptor complex [41].

In addition, it is thought that GOS can neutralize or interfere with bacterial toxins [10], as well as participate in ROS scavenging and peroxidase reduction [42], which might prevent the activation of NLRP3 inflammasome (**Figure 10**). Our observations indicate that GOS can inhibit *M. haemolytica* and/or LPS-induced mitochondrial peroxidation in both PBECs and A549 cells. The *in vitro* data from human A549 cells are in line with the data from bovine PBECs, showing the promising possibility of supplementing GOS in preventing human (respiratory) inflammation/infection.

In conclusion, NLRP3 inflammasome activation was observed in the airways of calves with lung infections, which may contribute to the elevated lung inflammation *in vivo* and may be associated with the activation of NLRP3 inflammasome in bronchial epithelial cells caused by *M. haemolytica* and its released LPS/leukotoxin. For the first time, the observed inhibitory effect of GOS on NLRP3 inflammasome activation brings us one step closer to the understanding of the anti-inflammatory mechanism of GOS, which could be important for their beneficial effect on respiratory infections.

Materials and methods

Animal experiment design

This experiment was conducted under the Dutch Law on Animal Experiments in accordance with EU Directive 2010/63 at the research facilities of the VanDrie Group (Scherpenzeel, The Netherlands) and was approved by the Animal Care and Use Committee of Wageningen University (AVD1040020185828,

Wageningen, The Netherlands).

The experiment consisted of 2 periods, experimental period, and growing period. Period 1 (experimental period) started when 150 male Holstein Friesian calves arrived at the experimental facilities (~18 days of age) and lasted from experimental week 1 till 8 in which GOS treatments were applied and most of the measurements were conducted on individual calves. At the end of period 1, 10 calves of each group were sacrificed, and bronchial mucosal tissue was stripped and collected from the primary bronchus. Period 2 lasted from experimental week 9 to slaughter at experimental week 27 and lung scores were performed in the slaughterhouse. In period 2, no oligosaccharide treatments were applied, and all calves received the same diet. Measurements and analyses were performed for all calves or for a subset of calves. The subset of calves included 2 calves per pen and 20 calves per group and was selected on body weight at arrival, closest to the average body weight of all calves at arrival.

During these periods, all calves were naturally exposed to pathogens in the environment. Individual antibiotic treatment was applied when required based on clinical signs of illness as assessed by the animal caretakers. The number of applied individual antibiotic treatments did not differ between the control and GOS intervention groups ($P > 0.1$). Group antibiotic treatment was applied equally to all groups if 10% of the calves had been treated within 5 days, or if 5% of the calves had become ill within 24h or when the situation required group antibiotics in the expert judgement of a veterinarian.

The *in vivo* study described in this article was part of a large calf trial, including 300 calves randomly assigned to a control group, 1% GOS group, 2% GOS group and 3 other groups with different (dietary) interventions (50 calves/group). In accordance with the purpose of this study, investigating the effect of oral GOS on lung infection, we reported the results of the analyses of the control and GOS groups (150 of 300 calves).

Animals, housing and feeding

Calves were housed in a mechanically ventilated stable throughout the experiment. The ambient lighting consisted of natural lighting plus artificial lighting from 0600 to 1800 h. Calves were housed in pens (9 m^2) containing wooden-slatted floors. In the first 6 weeks after arrival, individual housing was applied (1.2 m^2/calf) by placing stainless steel fences within the pens. After 6 weeks, the individual fencing was removed, and calves were housed in groups of five.

In period 1, 150 male Holstein Friesian calves (43.3 ± 0.26 kg, means ± SEM) of German origin were used and assigned randomly to 3 groups supplying with

calf milk replacer (MR) with or without GOS (Vivinal GOS syrup, FrieslandCampina Ingredients, The Netherlands) twice a day. The detailed composition of GOS is summarized in **Figure 1B**. The MR mainly contained 527 g/kg whey powder, 35 g/kg lactose, 52 g/kg delactosed whey powder, 50 g/kg whey protein concentrate, 60 g/kg soy protein concentrate, 50 g/kg soluble wheat protein, 3 g/kg pea fiber, 179.4 g/kg fat sources, 9.7 g/kg calcium formate, 2 g/kg citric acid, 4 g/kg sodium bicarbonate, 3.5 g/kg mono ammonium phosphate, 9.8 g/kg lysine, 2.4 g/kg methionine, 1.3 g/kg threonine, 0.2g/kg aroma and 10 g/kg premix. GOS administered via the MR were included at the expense of lactose, corrected for the purity and DM of the GOS products used. Group 1 as a control group included 50 calves and received MR without GOS. Group 2 and 3 included 50 calves and received MR containing 1% or 2% GOS, respectively.

Blood sampling and hematological analyses

Blood samples were collected of all calves by venipuncture in the jugular vein at arrival before the first MR feeding (baseline, week 0), and additionally at experimental week 2, 4 and 6 from 20 calves per group. Blood was collected in 9 mL and 4 mL K_2-EDTA tubes and was kept on ice for collection of plasma or kept at room temperature for analysis of leukocyte numbers the same day by fluorescence flow cytometry using a Sysmex 1800iV (Sysmex Europe GmbH, Norderstedt, Germany), respectively. Plasma was collected after centrifugation at 2,000 x *g* and 4°C for 20 min and was stored at -20°C pending further analyses.

BALF sampling and phenotyping

BALF was obtained by use of a technique adapted from Caldow *et al.* [43]. Briefly, a calf was restrained in the feeding fence and the head of calf was lifted and extended so that the nasal bone was parallel to the ground. Ethanol (70%) was used to clean the nose/nostrils of the calf. A sterilized 100 cm BAL catheter was inserted through a naris and blindly guided through the nasal passage into the trachea until the end was wedged in a bronchus. The correct placement of the catheter was verified by elicitation of the coughing reflex, the outstretch of the tongue, movement of air into and out of the catheter with each breath, and the absence of rumen contents, odor, and gurgling from the catheter. Once wedged in the appropriate location, a syringe was connected to the catheter and a total of 30 mL sterile saline (0.9% NaCl, 37°C) solution was slowly infused and immediately aspirated back into the syringe after each infusion. BALF (17.7 ± 0.4 mL, means ± SEM) was obtained from each calf and stored in a 50 mL tube on ice until further processing in the lab the same day.

Thereafter, BALF was filtered by passing through a 70 µm cell strainer (Corning, NY) to remove debris. To obtain cell pellets and perform cell counts,

BALF suspension was centrifuged (5 min, 400 x *g* at 4°C) and the remaining pellet was re-suspended in 1 mL cold FBS (4°C). After centrifugation, the supernatant was aliquoted into 1.5 mL tubes and stored at -80°C for further analysis. Cell number was determined by automatically counting in a Cellometer Bright Field cell counter (Nexcelom Bioscience, Lawrence, MA). For differential BALF cell counts, 0.5 x 10^6 BALF cells were used to make cytospins stained with Diff-Quick (Medion Diagnostics, Medion Diagnostics International Inc., Miami, FL) and a minimum of 400 cells were counted.

Lung scores

Calf lungs were scored and obtained by using a scoring system adapted from Leruste *et al.* [44]. Briefly, the observer (veterinarian) visually examined each lung (cranial and ventral lobes) evaluating signs of pneumonia. Each examined lung was classified according to a 4-point scale for pneumonia from healthy lung (score 0) to severe lesions (score 3). Score 0 for healthy lungs (pale orange color with no sign of pneumonia), score 1 for minimal or mild lesions (one spot of grey-red discoloration), score 2 for moderate lesions (one larger or several small spots of grey-red discoloration with a total surface of less than 1 lobe), and score 3 for severe lesions (grey-red discoloration area of at least one full lobe and/or presence of abscesses). The results were shown as a percentage of the total calves with different severity of pneumonia.

Clinical scores

Clinical scoring was performed weekly for all calves, according to the Wisconsin calf respiratory scoring system [45], in which a score from 0 to 3 was provided for rectal temperature, coughing, nasal discharge and behavior. The point scale used for respiratory clinical scoring was used as previously described (Chapter 3, Table 2). Clinical score was calculated as the sum of these 4 scores.

Identification of *M. haemolytica* in BALF and bronchial mucosal tissue

DNA was extracted from BALF and bronchial mucosal tissue using PureLink Genomic DNA Mini Kit (Invitrogen, Life Technologies, San Diego, CA) following the manufacturer's instructions. Real-time PCR methods for the detection of species-specific genes for *M. haemolytica* were performed using the primers and probes of BactoReal Kit (DVEB02911, Ingenetix GmbH, Vienna, Austria). BactoReal Kit detects the 16S rDNA gene of *M. haemolytica*. A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *M. haemolytica* specific DNA.

Assay mix was prepared in a 20 µL volume that contained 10 µL of DNA Reaction Mix, 3 µL PCR grade water, 5 µL extracted DNA from samples, 1 µL

primer, and 1 µL probe. Negative and positive controls were replaced by PCR grade water and positive *M. haemolytica*-DNA in the same kit, respectively. Real-time PCR was conducted on a Real-Time PCR Detection System (Bio-Rad, Hercules, CA).

Detection of *M. haemolytica*-LPS IgG levels in BALF and blood

M. haemolytica-LPS IgG levels in BALF and blood were measured according to manufacturer's instructions (BIO/K-139, Bio-X Diagnostics, Rochefort, Belgium). Negative and positive controls were provided by the same kit. The presence of *M. haemolytica*-LPS IgG was detected at 450nm using a microplate reader (Bio-Rad Laboratories) and showed as the fold of the positive control.

Isolation and culture of PBECs

Isolation and culture of PBECs were conducted as previously described [17]. Briefly, PBECs were isolated from bovine bronchial epithelium obtained from the lungs of freshly slaughtered calves aged 6-8 months provided by Ekro bv (Apeldoorn, The Netherlands). After digesting of the bronchial epithelium, PBECs were collected and grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and attached to collagen-coated plates in serum-free RPMI-1640 medium for 2-3 days until reaching near-confluence (70-90%) and then replaced with RPMI-1640 medium containing 10% FBS, 1% L-glutamine, 1% MEM NEAA, and 1% penicillin–streptomycin (Sigma-Aldrich, Zwijndrecht, The Netherlands) for future culture and experiments as we described before [17].

Human alveolar epithelial cell (A549) culture

Human Type II alveolar epithelial cells (A549; ATCC, Manassas, VA) were grown in Ham's F-12K Medium (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS and 1% penicillin–streptomycin (Sigma-Aldrich) in 5% CO_2 at 37°C.

Bacterial growth conditions

M. haemolytica (isolated from infected lungs of a pneumonic calf) was kindly provided by Prof. Jos van Putten (Department of Infectious Diseases and Immunology, Utrecht University, The Netherlands). *M. haemolytica* was incubated overnight at 37°C in 5% sheep blood agar (bioTRADING, Mijdrecht, The Netherlands).

PBECs and A549 treatments

PBECs were cultured at a density of 1 x 10^6 cells/mL in 96- or 6-well plates (Corning) pre-coated with collagen, fibronectin and BSA as described before [17]. After reaching near-confluence, these PBECs were pretreated with 0.5%, 1% or

2% GOS for 24h or pretreated with MCC950 (10 µM; InvivoGen, San Diego, CA) for 6h prior to stimulation with LPS (10 µg/mL; isolated from *E. coli O111:B4*, Sigma-Aldrich) for 6 or 24h with or without ATP (5 mM; InvivoGen) for 0.5h, or stimulation with leukotoxin A (10 ng/mL; Enzo Life Sciences, Bruxelles, Belgium) for 0.5, 1, 6, 12 or 24h or stimulation with *M. haemolytica* (1 x 10^5 CFU/mL) for 24h, or stimulation with rotenone (10 µM; Sigma-Aldrich) for 6h. After stimulation, supernatants were collected and stored at -20 °C until analysis.

A549 cells were cultured at a density of 0.5 x 10^5 cells/mL in 96- or 6-well plates (Corning). After reaching near-confluence, A549 cells were pretreated with 2% GOS for 24h prior to stimulation with LPS (10 µg/mL; *E. coli O111:B4*, Sigma-Aldrich) for 6 or 24h with or without ATP (5 mM; InvivoGen) for 0.5h. After stimulation, supernatants were collected and stored at -20 °C until analysis.

Caspase-1 activation assay

Caspase-1 activity in PBECs and A549 cells was determined in 50 µL cell lysates using a commercial kit (ab39412, Abcam, Cambridge, UK) according to the manufacturer's instructions. For the *in vivo* experiments, same weight of bronchial mucosal tissue was homogenized in lysis buffer from the kit, and 50 µL of the lysates was assayed following the manufacturer's instructions. Assays were performed in duplicate, and averages were taken. The results were shown as the percentage of control.

Lipid peroxidation and ROS measurements

Lipid peroxidation (malondialdehyde; MDA) in the PBECs, A549 cells, blood and bronchial mucosal tissue was measured using a commercial kit (ab118970, Abcam) according to the manufacturer's instructions. ROS in PBECs and A549 cells were assessed using the cell-permeant probe H_2DCFDA (MP36103, Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. The level of ROS was shown as the percentage of control.

ATP measurement

The production of ATP was measured using the ATP determination kit (A22066, Invitrogen, Thermo Fisher Scientific) following the manufacturer's instructions. Briefly, PBECs after different treatments were resuspended and gently mixed in reaction buffer containing 1 mM DTT, 0.5 mM luciferin, and 1.25 µg/mL luciferase, and readings were taken in a luminometer (GloMax, Promega Corp., Madison, WI).

LDH assay

PBECs were grown in 96-well plates as described above and the cytotoxic effect

of LPS, leukotoxin A or *M. haemolytica* on the PBECs was evaluated by measuring LDH leakage. LDH was measured in the supernatants using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega Corp.) according to manufacturer's instructions.

Thiazolyl blue tetrazolium bromide (MTT) assay

PBECs were grown in 96-well plates as described above and the viability of cells was measured using MTT assay. MTT (Sigma-Aldrich) was dissolved at a final concentration of 0.5 mg/mL in cell culture medium. Each culture well was delicately washed with pre-warmed PBS before adding a 120 µL MTT solution. After 3h incubation (37 °C, 5% CO2), the formed formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) and absorbance was read at 595 nm using a microplate reader (Bio-Rad).

ELISA measurement

Levels of IL-8 (Mabtech, Nacka Strand, Sweden), IL-6 (Invitrogen, Thermo Fisher Scientific), IL-1β (Invitrogen, Thermo Fisher Scientific) and/or TNF-α (R&D Systems, Minneapolis, MN) in the BALF and blood of calves and in the supernatants of PBECs were determined by using ELISA kits according to manufacturer's instructions. Levels of IL-1β (BioLegend, San Diego, CA) in the supernatants of A549 cells after different treatments were also measured by using the ELISA kits. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad).

Western blotting

Cell lysates of PBECs and tissue lysates of calves after different treatments were prepared by adding RIPA cell lysis buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors (Roche Applied Science, Pennsburg, Germany). Total protein content was estimated by bicinchoninic acid analysis (Pierce, Thermo Fisher Scientific) according to the manufacturer's protocol. Samples were loaded onto polyacrylamide gradient gels (4-20% Tris-HCl, Bio-Rad) and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with PBS containing 0.05% Tween-20 (PBST) and 5% milk proteins for 1h at room temperature and incubated with primary antibodies at 4 °C overnight (NLRP3, 1:1000, PA5-18118; TLR4, 1:1000, PA5-23284, Thermo Fisher Scientific; IL-1β, 1:100, MCA-1658, Bio-Rad; p-p38, 1:1000, #9215; p-ERK1/2, 1:1000, #9101; p-JNK1/2, 1:1000, #9251; p-p65, 1:1000, #3033; β-actin, 1:5000, #4970, Cell Signaling Technology, Beverly, MA), followed by washing blots in PBST. Appropriate horseradish peroxidase-coupled secondary antibodies from Dako (Agilent Technologies, Santa Clara, CA) were

applied for 1h. Membranes were incubated with ECL western blotting substrates (Bio-Rad) prior to obtaining the digital images. Digital images were acquired with the Molecular Imager (Gel DocTM XR, Bio-Rad) and analyzed with Image lab 5.0 (Bio-Rad).

Immunofluorescence

PBECs were grown in 6-well plates as described above and detected for the NLRP3 protein using immunofluorescence. PBECs were fixed with 10% formalin (Baker, Deventer, The Netherlands) and after washing with PBS, the cells were permeabilized with PBS containing 0.1% (v/v) Triton-X-100 for 5 minutes, followed by blocking with 5% serum in 1% (w/v) BSA/PBS for 30 minutes at room temperature. Thereafter, PBECs were incubated overnight with primary antibodies NLRP3 (1:50, ab4207, Abcam) followed by incubation with Alexa-Fluor conjugated secondary antibodies (Invitrogen) for 1h at room temperature in the dark. Nuclear counterstaining was performed with DAPI containing anti-fade reagent (ready to use, Invitrogen). NLRP3 were visualized and images were taken using the Keyence BZ-9000 (KEYENCE Corporation, Osaka, Japan).

Statistical analysis

Experimental results *in vivo* are expressed as non-transformed means ± SEM. *In vivo* data were analyzed for treatment and time effects with SAS 9.4 (SAS Institute Inc., Cary, NC), using the MIXED procedure, including time as a random statement with calf as unit. For each parameter, the covariance structure was selected based on the lowest AIC and BIC. All analyses included a random effect of pen. For leukocyte counts, the concentration/percentage at arrival (before application of the treatments) was included as a co-variable in the model. Studentized residuals of each model were checked visually on the homogeneity of variance and data were transformed if required to obtain homogeneity of variance. To evaluate differences between treatments, the contrast statement was used, and treatment differences were assessed per timepoint separately. Clinical scores were assessed for treatment and time effects using the GLIMMIX procedure with a multinomial distribution including a random pen effect and potential differences between the treatments were evaluated using the contrast statement per timepoint. The Chi-square test was performed for the proportion of different lung lesions and the positivity of *M. haemolytica* in calves. Differences were considered significant when $P < 0.05$ and considered a trend when $P < 0.10$.

Data from *in vitro* experiments are determined by one-way ANOVA or two-way ANOVA followed by Tukey with selected comparisons as a *post hoc* test when F achieved $P < 0.05$ and there was no significant variance in homogeneity. All experimental results are expressed as means ± SEM and analyzed using the

GraphPad Prism version 7.0 software (San Diego, CA). Results were considered statistically significant when $P < 0.05$ and considered a trend when $P < 0.10$.

Abbreviations: ATP, adenosine triphosphate; BALF, broncho-alveolar lavage fluid; COS, chitosan-oligosaccharides; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides; HMOs, human milk oligosaccharides; IL-1, interleukin-1; LDH, lactate dehydrogenase; LPS, lipopolysaccharides; MR, milk replacer; MDA, malondialdehyde; MTT, thiazolyl blue tetrazolium bromide; MAPK, mitogen-activated protein kinase; NLRP3, NLR family pyrin domain containing 3; NDOs, Non-digestible oligosaccharides; NF-κB, nuclear factor kappa B; NAC, acetylcysteine; ROS, reactive oxygen species; PBECs, primary bronchial epithelial cells; RCrL, right cranial lobe; TLR4, Toll-like receptor 4.

Funding information: This work was funded by the Netherlands Organisation for Scientific Research (NWO), grant number: ALWCC.2015.4. Research grant funding was received from the China Scholarship Council for Y. Cai.

Author contribution: Y.C. and S.B. conceptualized the study; S.B., G.F., M.S.G., and W.J.J.G. advised on study design; Y.C. and M.S.G. directed experiments, analyzed data, performed statistical analysis; Y.C. wrote the manuscript, S.B. and G.F. edited the manuscript. Y.C., S.B., G.F., M.S.G., and W.J.J.G. gave final approval of the version to be published.

Supplementary information

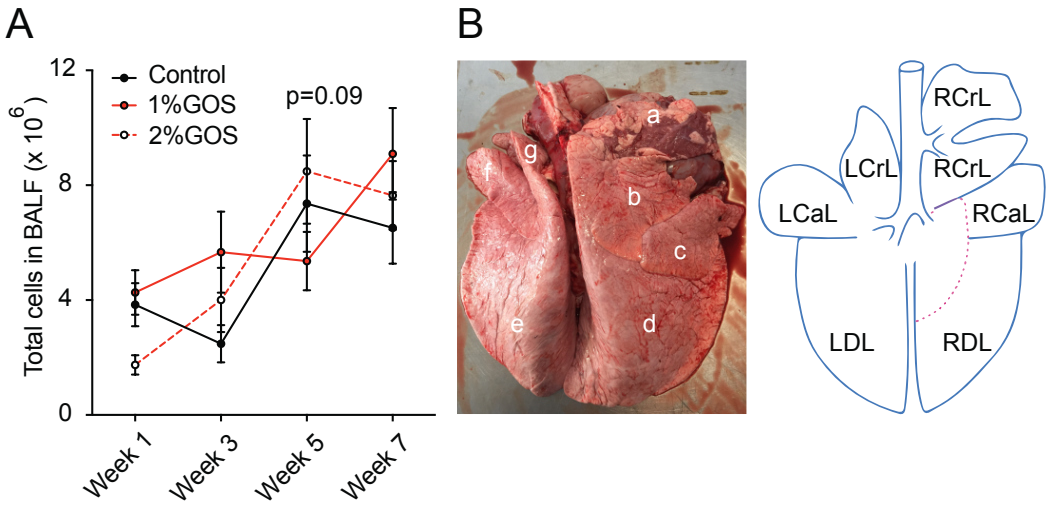


Figure S1. Effect of GOS on total cell numbers in BALF and infected calf lungs (lobes). (**A**) Number of total cells in BALF was measured at week 1, 3, 5 and 7 (n=60, 20 calves/group). (**B**) Infection foci was observed in RCrL of control calf lungs at week 8. a and b = RCrL (right cranial lobe); c = RCaL (right cardiac lobe); d = RDL (right diaphragmatic lobe); e = LDL (left diaphragmatic lobe); f = LCaL (left cardiac lobe); g = LCrL (left cranial lobe). P=0.09 (control week 5 vs week 1). Data are presented as means ± SEM. BALF = broncho-alveolar lavage fluid; GOS = galacto-oligosaccharides.

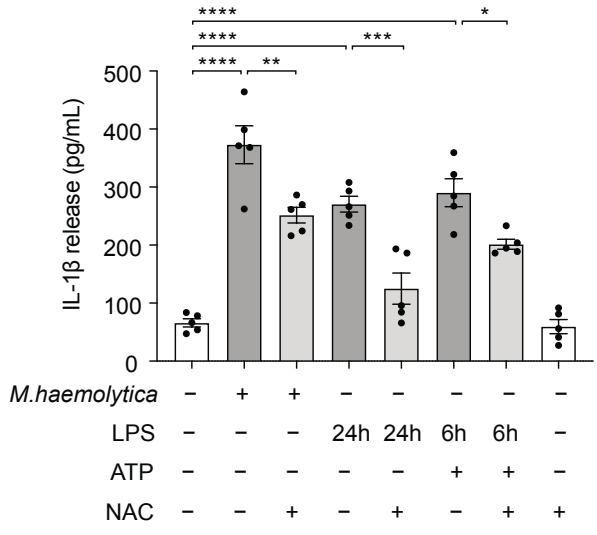


Figure S2. NAC inhibit *M. haemolytica*/LPS/ATP-induced IL-1β release in primary bronchial epithelial cells. PBECs were incubated with *M. haemolytica* (1 x 10^5 CFU/mL, 24h) or LPS (10 µg/mL, 24h) or LPS+ATP (10 µg/mL + 5 mM, 6h + 0.5h) with or without 6h pretreatment with 1mM NAC. The IL-1β release was determined by ELISA in the supernatants. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves). ATP = adenosine triphosphate; GOS = galacto-oligosaccharides; LPS = lipopolysaccharide; NAC = acetylcysteine; PBECs = primary bronchial epithelial cells.

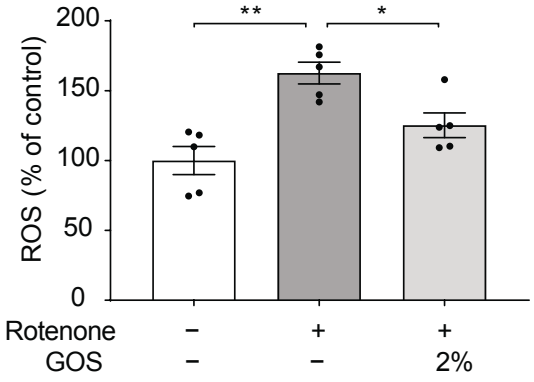


Figure S3. GOS inhibit rotenone-induced production of mitochondrial ROS in primary bronchial epithelial cells. PBECs were incubated with 10 μM rotenone for 6h with or without 24h GOS pretreatment. The production of mitochondrial ROS in PBECs was assessed and data were shown as a percentage of control. **P*<0.05; ***P*<0.01. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves). GOS = galacto-oligosaccharides; ROS = reactive oxygen species; PBECs = primary bronchial epithelial cells.

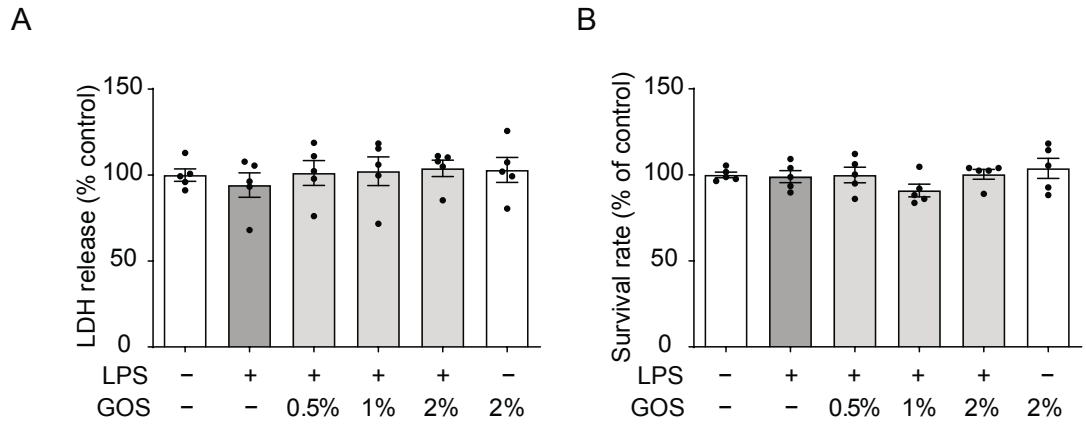


Figure S4. GOS do not affect LDH release and survival rates in primary bronchial epithelial cells. PBECs were treated with 10 μg/mL LPS for 24h with or without 24h GOS pretreatment. (**A**-**B**) LDH release was measured in the supernatants of PBECs, and survival rates were determined by the percentage of MTT levels in PBECs. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves). GOS = galacto-oligosaccharides; LDH = lactate dehydrogenase; LPS = lipopolysaccharide; MTT = thiazolyl blue tetrazolium bromide; PBECs = primary bronchial epithelial cells.

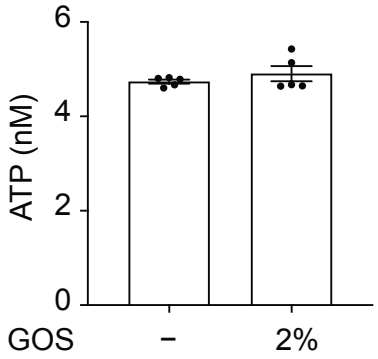


Figure S5. GOS alone do not affect ATP production in primary bronchial epithelial cells. PBECs were treated with or without 48h GOS, then ATP production in PBECs was assessed and data were shown as an absolute amount. Data are presented as means ± SEM. Figure S5 has the same control group as Figure 6D. All data shown are representative of five independent experiments (n=5 donor calves). ATP = adenosine triphosphate; GOS = galacto-oligosaccharides; PBECs = primary bronchial epithelial cells.

Table S1. The number of positive for *M. haemolytica* in BALF of control calves.

BALF samples of control calves (n=20 calves)	Week 1	Week 3	Week 5	Week7
M. haemolytica-LPS IgG positivity	0	4	16	16
p-value (vs week1)	—	0.11	<0.0001	<0.0001

[1]BALF, broncho-alveolar lavage fluid; LPS, lipopolysaccharide.
[2]The present Table S1 is the same as Table 1 in Chapter 3

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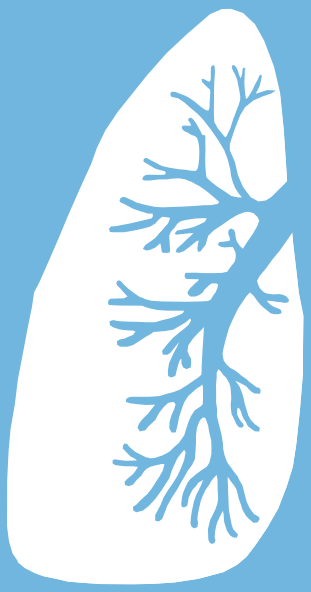

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Chapter 5

Galacto-oligosaccharides as an Anti-bacterial and Anti-invasive Agent in Lung Infections

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Biomaterials (Revision)

Abstract

Emerging antimicrobial resistance in respiratory pathogens asks for novel intervention strategies. We investigated whether galacto-oligosaccharides (GOS) with nutraceutical potential may prevent infection related inflammation *in vitro* and protect calves against lung infections *in vivo*. We found that 8% and 16% GOS significantly reduced the viability of *Mannheimia haemolytica* (one of the major *Pasteurellaceae* associated with bovine lung infections) *in vitro*, while 4% GOS mainly lowered the growth of *M. haemolytica*. This might be related to an increased bacterial cell membrane permeability by GOS. In addition, 2% GOS decreased the *M. haemolytica*-induced IL-8 and IL-6 release and prevented barrier dysfunction in primary bronchial epithelial cells (PBECs), which might be related to the downregulation of "TLR4/NF-κB" pathway and the anti-invasive and anti-adhesion effects of GOS. Interestingly, intranasal application of GOS partly suppressed different inflammatory mediators in blood and bronchoalveolar lavage fluid and decreased the positivity of *M. haemolytica* in the lungs of calves. This study describes for the first time that GOS can exert an anti-bacterial effect and prevent adhesion to and invasion of PBECs by *M. haemolytica*. Besides, we showed that GOS might be used as an intranasal application in lung infections.

Keywords: Oligosaccharides; Respiratory infections; Bacteriostatic effect; Airway inflammation; *Mannheimia haemolytica*; Primary bronchial epithelial cells

Introduction

Lung infections caused by bacteria are a common health problem in all domestic animal species as well as in humans. Pneumonia alone is responsible for more than 1.3 million child deaths annually [1]. Respiratory pathogens can successfully colonize the epithelial lining of the upper respiratory tract, grow on the mucosal surface, escape from the host immune response, and spread to a susceptible host [1, 2]. These pathogens may invade the lower respiratory tract and induce infections in susceptible hosts with a weakened immune system and/or environmental stress, including adverse climatic conditions, crowding and indoor air pollution, can serve as cofactors in the pathogenesis of lung infections [2]. The calf is considered to be an excellent animal model for studying lung infections: infections are well-characterized, calves and humans share similar clinical manifestations (e.g., cough reflex) and anatomical features (e.g., bronchial glands, cartilage airways), and the prevalence of pneumonia is extremely high, involving the most important airborne and close contact transmission [2].

Mannheimia haemolytica is a Gram-negative bacterium and is one of the major *Pasteurellaceae* associated with bovine lung infections [3, 4]. *M. haemolytica* can colonize the upper respiratory tract and replicate explosively, afterwards invade deeper into the lower respiratory tract and infect alveolar lining cells [2, 5]. *M. haemolytica* can impair the airway epithelial barrier function and induce proinflammatory mediator release, which cause an acceleration of invasion. For example, *M. haemolytica* released lipopolysaccharide (LPS), a potent virulence factor, can activate the Toll-like receptor 4/nuclear factor-κB (TLR4/NF-κB) pathway, leading to IL-8 and IL-6 release [5-7].

Non-digestible oligosaccharides (NDOs) are a group of complex carbohydrates with the potential to prevent respiratory infections [8, 9]. Oral administration of NDO mixtures containing galacto-oligosaccharides (GOS) and long-chain fructo-oligosaccharides (lcFOS) reduce, especially respiratory, infections in first six months of life [10], and decrease the frequency of respiratory infections, fever, and antibiotic use during the first two years of life [11]. In addition to the indirect effects of reducing respiratory infections (e.g., via the gut-lung axis) [12], NDOs can also be used as a direct anti-bacterial agent [13], for instance, human milk oligosaccharides (HMOs) can directly inhibit the growth of group B *Streptococcus* (GBS) *in vitro* [14].

Here, we hypothesize that GOS may alleviate lung infection-induced inflammation *ex vivo* and *in vivo*. We showed for the first time that intranasal GOS suppressed airway inflammation in calves with lung infections, which might be related to the interesting findings that GOS lowered *M. haemolytica* growth and

viability as well as prevented *M. haemolytica* from invading primary bronchial epithelial cells (PBECs) and from disrupting the airway epithelial barrier. NDOs, such as GOS, with or without the combination of standard drugs might be helpful in resisting inflammation caused by lung infections and we innovatively propose a new route of administration (intranasal application) for NDOs to treat these infections.

Results

Effect of GOS on the growth of *M. haemolytica in vitro*

M. haemolytica (1 x 10^5 CFU/mL) was incubated with increasing concentrations of GOS for 0 to 24h. Bacterial growth assays (OD600nm) in the presence of different concentrations of GOS (4%, 8%, and 16%) revealed a significant concentration-dependent reduction in growth of *M. haemolytica*. The minimum inhibitory concentration of GOS was 8% (**Figure 1A**). In addition, **Table S1** showed that different concentrations of GOS did not change the pH of the medium after 24h incubation in the absence of *M. haemolytica*.

To determine whether GOS was bacteriostatic or bactericidal, supernatants from *M. haemolytica* at 0h and 24h with or without GOS treatment were sub-cultured onto sheep blood agar plates. *M. haemolytica* treated with 8% and 16% GOS for 24h exhibited significant lower CFU/mL compared to *M. haemolytica* alone at 24h, indicating inhibition of growth (**Figure 1B**). In addition, the visible/surviving *M. haemolytica* on the agar plates was 0.82 x 10^5 CFU/ml at 0h, which is 82% of the expected CFUs (1 x 10^5 CFU/mL) of *M. haemolytica* based on the absorbance values (**Figure 1C**). Incubation with 8% and 16% GOS treatments significantly lowered the viability of *M. haemolytica*, whereas only 8% and 0.5% of the initial *M. haemolytica* (t=0 h) survived, respectively (**Figure 1C**).

The LIVE/DEAD BacLight assay was used to investigate the effects of GOS on bacterial viability and membrane permeability. Compared to *M. haemolytica* at 0h, the live/dead ratio of *M. haemolytica* at 24h did not significantly change (**Figure 1D**). The live/dead ratio of *M. haemolytica* at 0h and 24h is close to 4, while 4%, 8% and 16% GOS-treated *M. haemolytica* showed lower ratios (**Figure 1D** and **E**). Especially, 8% and 16% GOS-treated *M. haemolytica* showed live/dead ratios lower than 0.5, indicating that the vast majority of *M. haemolytica* present was dead after GOS incubation, which is in line with the CFU/mL counted on the agar plates depicted in **Figure 1B** and **C**. In addition, 8% and 16% GOS-treated *M. haemolytica* showed a significantly higher percentage of impaired membrane than *M. haemolytica* at 24h (**Figure 1F**).

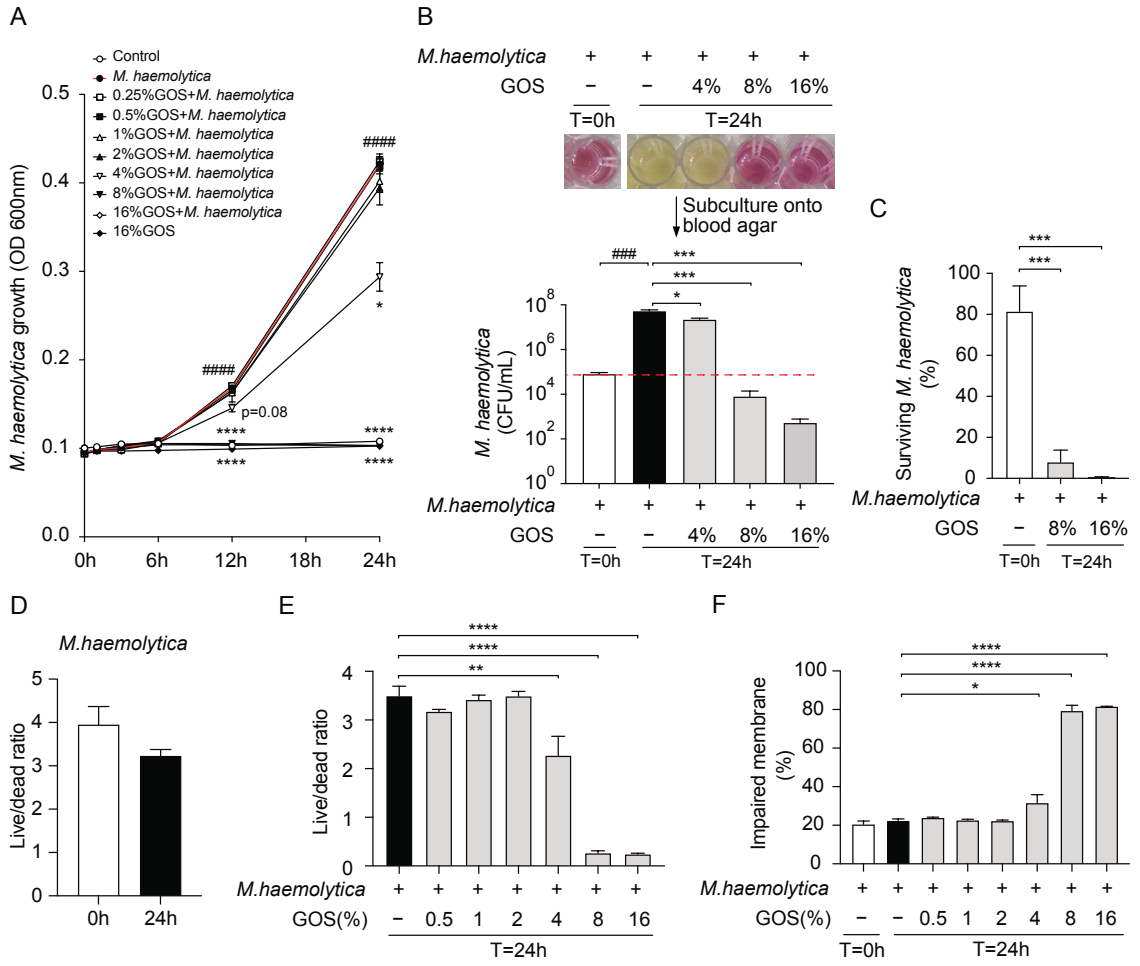


Figure 1. Effect of GOS on the growth of *M. haemolytica in vitro*. *M. haemolytica* (1 x 10^5 CFU/mL based on the absorbance values) was treated with or without different concentrations of GOS for 0 to 24h, thereafter supernatants from 0h and 24h were sub-cultured onto 5% sheep blood agar plates overnight. (**A**) *M. haemolytica* growth (OD600nm) was recorded at different timepoints (0, 1, 3, 6, 12 and 24h) after GOS treatments. (**B-C**) *M. haemolytica* numbers at 0h and 24h after GOS treatments were determined by counting CFUs on each agar plate and calculated as CFU/mL. Surviving *M. haemolytica* at 0h and 24h was represented as the percentage relative to the expected CFUs (1 x 10^5 CFU/mL) of *M. haemolytica* based on the absorbance values. (**D**) Live/dead ratio of *M. haemolytica* at 0h and 24h was determined by a LIVE/DEAD BacLight assay. (**E**) Live/dead ratio of *M. haemolytica* at 24h with or without the treatment of different GOS concentrations was determined by a LIVE/DEAD BacLight assay. (**F**) The percentage of *M. haemolytica* with impaired membrane integrity was determined at 0h and 24h after GOS treatments. ###*P*<0.001; ####*P*<0.0001 (*M. haemolytica* 12 or 24h vs 0h); **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 (GOS-treatment vs *M. haemolytica* group). Data are presented as means ± SEM. All data shown are representative of at least three independent experiments (n=3-6 bacterial generations, 1-2 generations per experiment).

Inhibitory effect of GOS in a bovine PBEC infection

To better understand the effect of GOS in the complex setting of bronchial epithelial cells, we isolated and cultured PBECs of healthy bovine lungs and developed an *ex vivo* infection model [7].

Cellular survival (MTT assay) and lactate dehydrogenase (LDH) assay showed that 2% GOS might be the optimal concentration to be administrated in this *ex vivo* infection model (**Figure 2A** and **B**). Although pretreatment with 2% GOS did not affect the *M. haemolytica* growth in the supernatant of PBECs (**Figure 2C**), infection assays showed that 2% GOS caused a significant decrease in adhesion to and invasion of (entry into) the cells by *M. haemolytica* (**Figure 2D** and **E**).

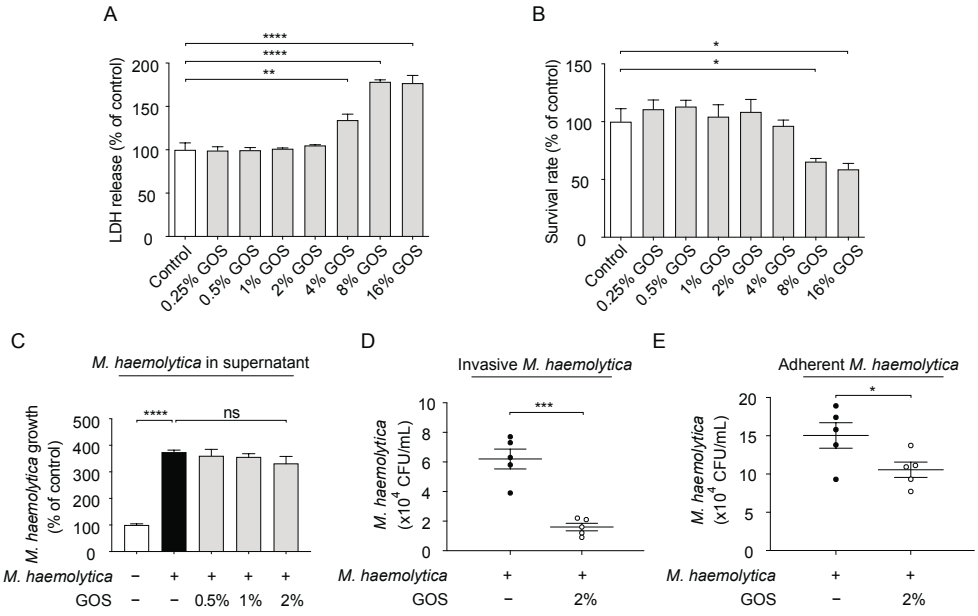


Figure 2. Inhibitory effect of GOS in a bovine PBEC infection. (**A**-**B**) PBECs were incubated with different concentrations of GOS for 48h. LDH release was measured in the supernatants of PBECs, and survival rates were determined by the percentage of MTT levels in PBECs. (**C**-**E**) PBECs were treated with *M. haemolytica* (1 x 10^5 CFU/mL) for 24h with or without 24h pretreatment with GOS, PBECs were lysed with or without supplementation of extracellular gentamicin (for killing the adherent *M. haemolytica*) and inoculated onto 5% sheep blood agar plates overnight. (**C**) *M. haemolytica* growth in the supernatants was determined by measuring turbidity of supernatants at 600nm and represented as a percentage of control (only medium). (**D-E**) The number of invasive (**D**) and adherent (**E**) *M. haemolytica* was determined by counting CFUs on each plate and represented as CFU/mL. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns=no significance. Data are presented as means ± SEM. All data shown are representative of three independent experiments (n=3 or 5 donor calves, 1-2 donor calves per experiment).

GOS inhibit *M. haemolytica*-induced inflammation and barrier dysfunction in PBECs

To learn more about the effect of GOS on the inflammatory response of the PBECs, we measured chemokine/cytokine levels in the supernatants of *M. haemolytica*-treated PBECs with or without preincubation with GOS. Release of IL-8 and IL-6 induced by *M. haemolytica* was significantly inhibited by pretreatment with GOS (1% and 2% GOS, **Figure 3A** and **B**), while the cellular survival and LDH release were not affected (**Figure S1A** and **B**).

Another characteristic of *M. haemolytica*-treated bronchial epithelial cells is the alterations in barrier function-associated molecules [7, 15]. In the present study, 1 x 10^5 CFU/mL of *M. haemolytica* exposure 24h resulted in a significant decrease in protein and fluorescence level of the tight junction protein ZO-1 and adherens junction protein E-cadherin in PBECs (**Figure 3C-E**). Moreover, the 24h pretreatment with 2% GOS prevented this reduction of ZO-1 and E-cadherin in protein level and fluorescence intensity (**Figure 3C-E**).

As a consequence of alterations in the function and expression of tight and adherens junction proteins, *M. haemolytica* is likely to impair transepithelial electrical resistance (TEER) of epithelial cell monolayers and to increase paracellular tracer transport. In the present study, PBECs exposed to *M. haemolytica* resulted in a significant decrease in TEER (**Figure 4A**), which facilitated the translocation of lucifer yellow from the apical to the basolateral compartment and this coincided with the release of IL-8 and IL-6 (**Figure 4B-D**). Interestingly, pretreatment with GOS prevented the decreased TEER and increased lucifer yellow flux and corresponding IL-8 and IL-6 release compared to *M. haemolytica*-treated PBECs (**Figure 4**).

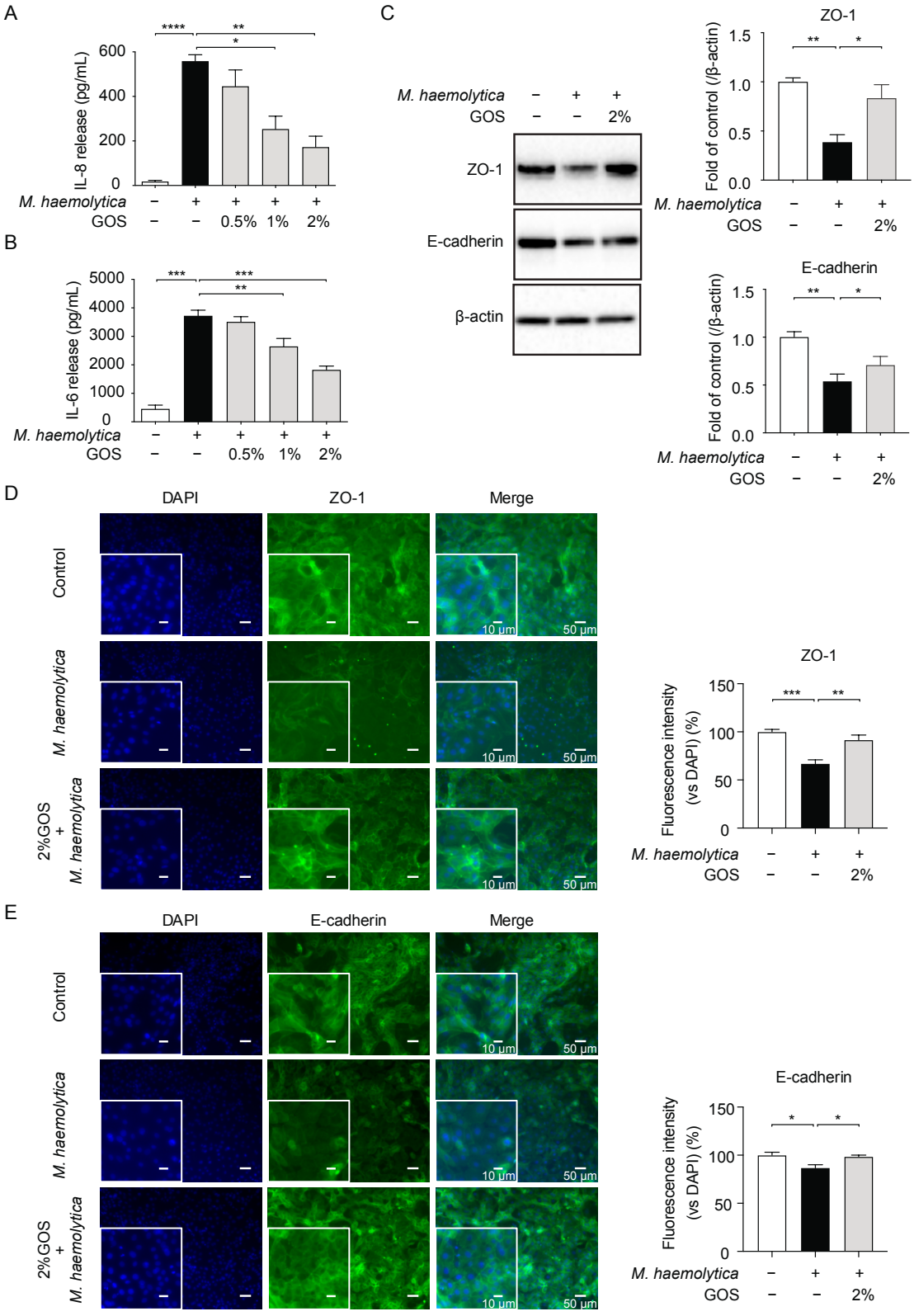


Figure 3. GOS prevent *M. haemolytica*-induced inflammation and alterations of barrier function-associated molecules in PBECs. PBECs were incubated with *M.*

haemolytica (1 x 10^5 CFU/mL) for 24h with or without pretreatment with GOS. (**A**-**B**) IL-8 and IL-6 levels in the supernatants of PBECs were measured by ELISA. (**C**) The immunoblots were obtained with ZO-1, E-cadherin and β-actin (protein loading control). (**D**-**E**) Cellular expression of ZO-1 and E-cadherin in PBECs was assessed by immunofluorescent staining and quantified as a percentage of fluorescence intensity. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of three independent experiments (n=3 or 5 donor calves, 1-2 donor calves per experiment).

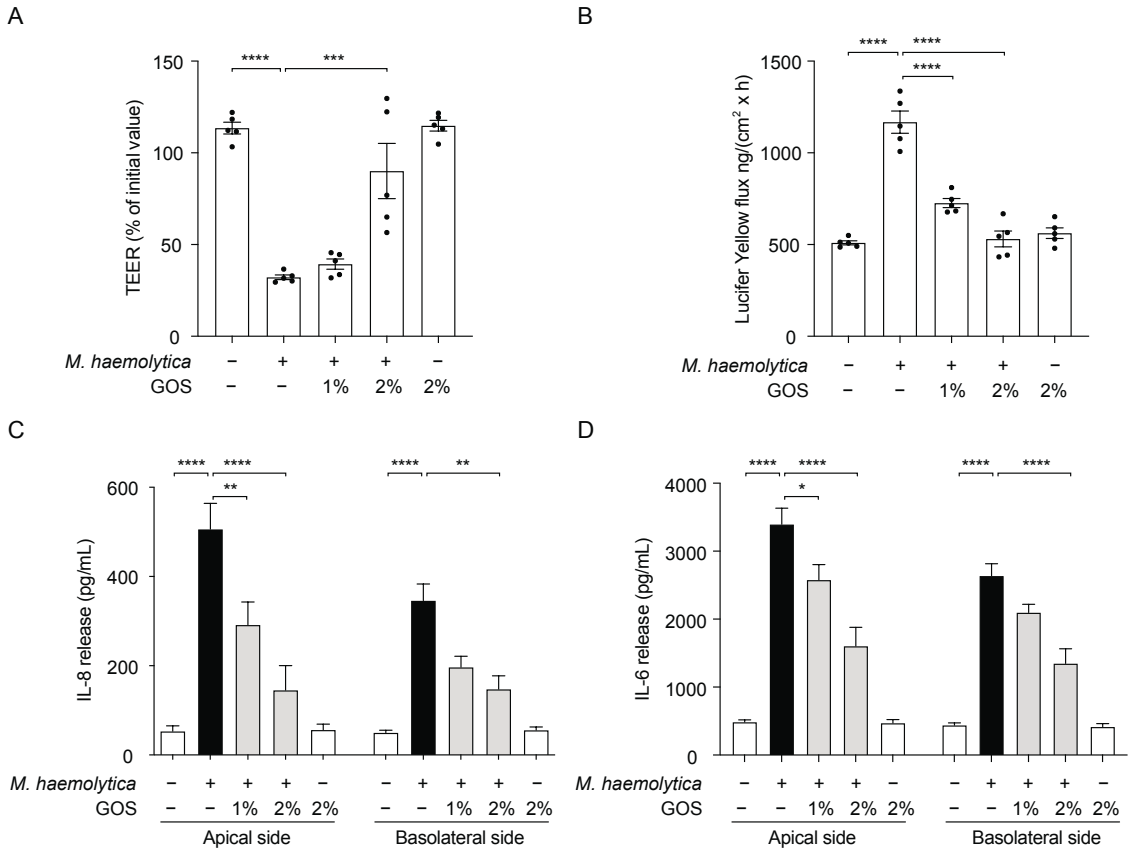


Figure 4. GOS prevent the *M. haemolytica*-induced disruption of the PBEC monolayer integrity. PBECs were grown on inserts and exposed to *M. haemolytica* (1 x 10^5 CFU/mL) at the apical compartment with or without GOS pretreatment at the apical and basolateral compartments. After 24h exposure, (**A**) TEER was measured and (**B**) lucifer yellow flux from apical to basolateral compartment was determined. The data of control and *M. haemolytica* groups in Figure 4A and B were reused in the control and *M. haemolytica* groups in Figure 5A and B of Chapter 6. (**C**-**D**) The release of IL-8 and IL-6 in both apical and basolateral compartment was assessed by ELISA. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves, one donor calf per experiment).

GOS and CLI-095 prevent *M. haemolytica*-induced activation of TLR4/NF-κB pathway and subsequent inflammation in PBECs

The activation of the TLR4/NF-κB signaling pathway played a possible role in the *M. haemolytica* and LPS-induced inflammation in our previous study [7]. Here, the pretreatment with 2% GOS showed a decrease in protein expression of TLR4 and phosphorylation of NF-κB p65 in PBECs after *M. haemolytica* exposure (**Figure 5A**).

A TLR4 inhibitor (CLI-095) was applied to investigate whether inhibition of the TLR4/NF-κB pathway could affect *M. haemolytica*-induced inflammation. As expected, CLI-095 significantly decreased the protein expression of TLR4 and p-p65 and the release of IL-8 and IL-6 in *M. haemolytica*-treated PBECs, while CLI-095 alone had no effects (**Figure 5**). Although CLI-095 inhibited IL-8 and IL-6 release, it did not prevent the *M. haemolytica*-induced disruption of the PBEC monolayer integrity (**Figure S2A** and **B**).

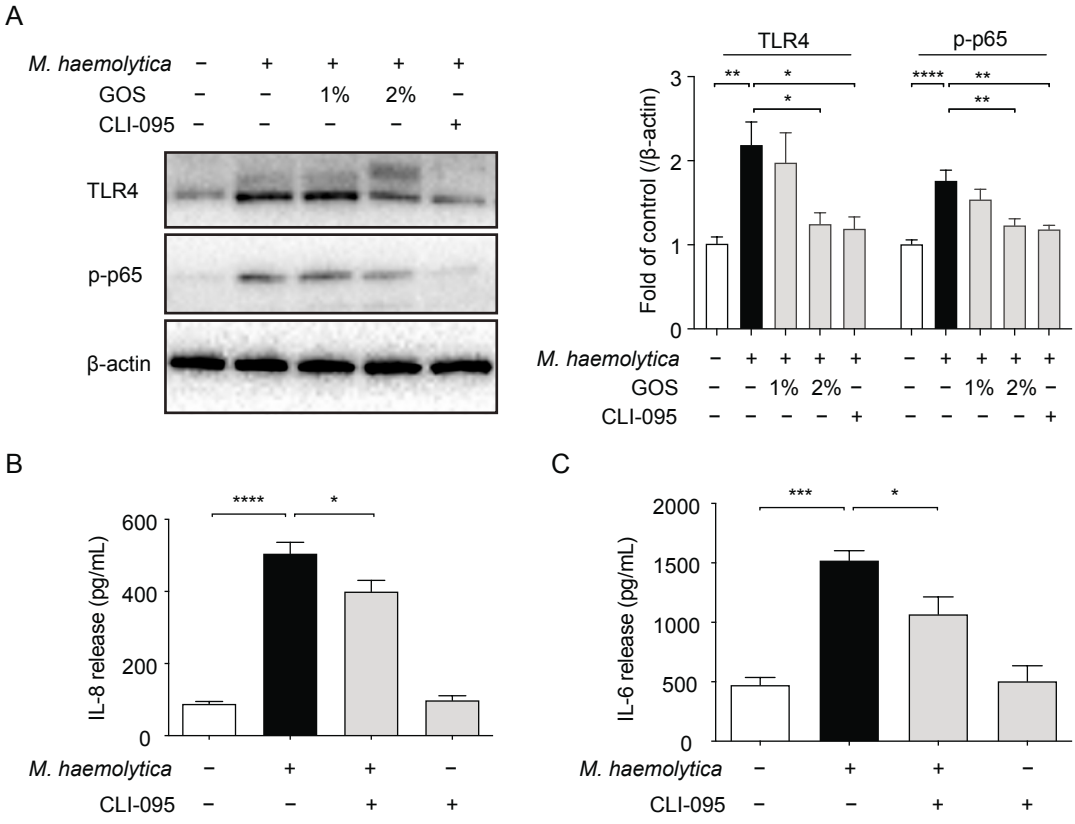


Figure 5. GOS and CLI-095 prevent *M. haemolytica*-induced activation of TLR4/NF-κB pathway and subsequent inflammation in PBECs. PBECs were incubated with *M. haemolytica* (1 x 10^5 CFU/mL) for 24h with or without GOS (24h) or CLI-095 (1μM; 3h)

pretreatment. (**A**) The immunoblots were obtained with TLR4, p-p65 and β-actin (protein loading control). (**B**-**C**) IL-8 and IL-6 levels in the supernatants of PBECs were measured by ELISA. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of three independent experiments (n=3 donor calves, one donor calf per experiment).

GOS prevent LPS- but not flagellin-induced inflammation in both bovine PBECs and human alveolar epithelial cells

Airway epithelial cells can recognize bacterial virulence factors (e.g., LPS and flagellin) through pattern-recognition receptors to initiate inflammation signaling [16]. Here, we assessed the effect of GOS on LPS- and flagellin-induced inflammatory responses in both bovine and human airway epithelial cells. In line with our previous findings [7], LPS and flagellin significantly increased the release of IL-8 and IL-6 in PBECs (**Figure 6A-D**). Despite a significant inhibition in LPS-induced IL-8 and IL-6 release caused by GOS pretreatment, GOS did not affect flagellin-induced IL-8 and IL-6 release (**Figure 6A-D**).

Comparable to the results with the PBECs, pretreatment with GOS significantly inhibited LPS, but not flagellin-induced IL-8 and IL-6 release from human alveolar epithelial (A549) cells (**Figure 6E** and **F**). Furthermore, LPS/flagellin with and without GOS supplementation did not affect the cellular survival and LDH release in both PBECs and A549 cells (**Figure S1C-F**).

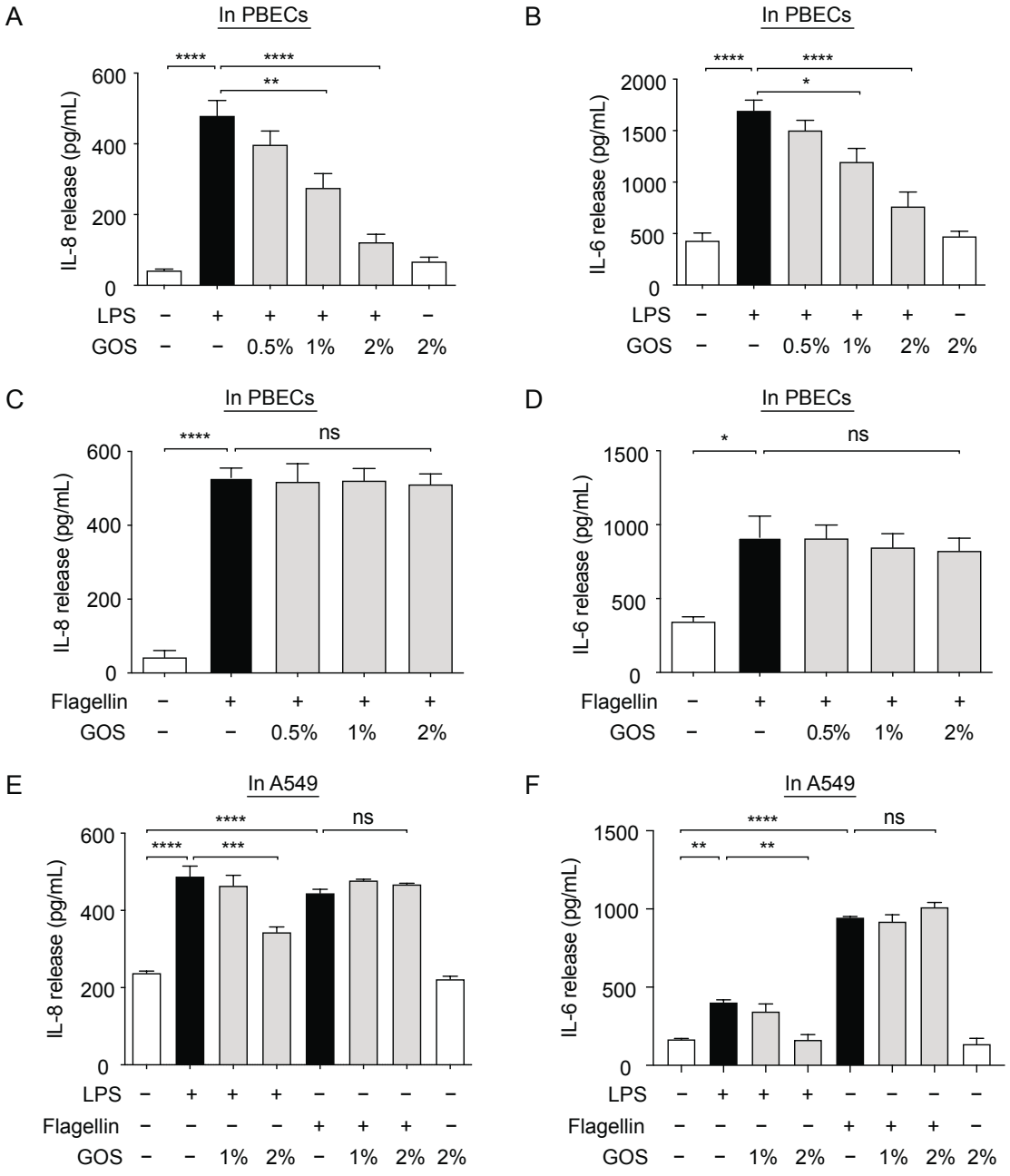


Figure 6. GOS prevent LPS- but not flagellin-induced inflammation in both bovine PBECs and human alveolar epithelial (A549) cells. PBECs and A549 cells were incubated with LPS (10 µg/mL) or flagellin (10 ng/mL) for 24h with or without GOS pretreatment. (**A**-**D**) Levels of IL-8 and IL-6 in the supernatants of PBECs were measured by ELISA (n=3 or 5 donor calves, 1-2 donor calves per experiment). (**E**-**F**) IL-8 and IL-6 levels in the supernatants of A549 cells were determined by ELISA (n=3 cell generations, one generation per experiment). **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns=no significance. Data are presented as means ± SEM. All data shown are representative of three independent experiments.

Intranasal application of GOS suppresses inflammation in calves with naturally occurring lung infections

Due to the interesting *in vitro* anti-bacterial, anti-adhesion and anti-inflammatory properties of GOS mentioned above, an animal experiment was conducted to investigate the effect of intranasal GOS administration in calves with naturally occurring lung infections. GOS-spray were intranasally administrated once per day from experimental week 1 till 8, thereafter these calves were not exposed to GOS from week 9 till 27 (**Figure 7A**).

An increase in total cell numbers (increased by 0.92-fold; mainly pulmonary leukocytes; **Figure S3B**) and neutrophil (increased by 11-fold) and lymphocyte (increased by 138-fold) numbers (**Figure S3C** and **E**) and percentages (**Figure 7D** and **E**) were observed in bronchoalveolar lavage fluid (BALF) of control calves from week 5, compared to week 1. In contrast, a significant decrease in macrophage numbers (decreased by 56%; **Figure S3D**) and percentages (**Figure 7C**) was found in BALF of control calves. At week 6, the blood leukocytes were increased by 26% in comparison with week 0 (baseline) (**Figure 7F** and **Figure S3A**). In addition, the clinical scores of control calves continuously increased over time and reached peak scores at week 6 (**Figure 7B**). These findings indicate that lung infections were present in these calves from week 5 [17].

Intranasal GOS had no effects on clinical scores from week 1 to 7 (**Figure 7B**). Intranasal GOS did not affect the total cell numbers (**Figure S3B**) and the number and percentage of lymphocytes (**Figure S3C** and **Figure 7E**) in BALF over time. However, intranasal GOS tended to increase the percentage and number of macrophages (**Figure 7C** and **Figure S3D**), tended to decrease the number of neutrophils (**Figure S3E**), and significantly decreased the percentage of neutrophils in BALF at week 5 (**Figure 7D**). After 6 weeks of natural exposure, GOS-spray significantly lowered the increase of blood leukocytes (**Figure 7F**), while tended to decrease the concentrations of leukocytes (**Figure S3A**).

The inflammatory response in the lungs was investigated by measuring cytokines/chemokines in BALF. TNF-α concentrations of control calves increased significantly over time and reached the highest-level during week 5. As expected, intranasal GOS suppressed TNF-α levels in BALF at week 5 (**Figure 7H** and **Figure S3F**). In addition, other cytokine/chemokine levels (IL-8, IL-6, and IL-1β) were also measured during week 5, indicated as timepoint for the lung infections. Intranasal GOS significantly reduced the concentrations of IL-6 and IL-1β (**Figure 7I** and **J**), but not IL-8 at week 5 (**Figure 7G**). The same cytokines/chemokines were analyzed in blood to investigate the effect of intranasal GOS on systemic

inflammation caused by lung infections at week 4 and 6 (one week prior to or one week after week 5). In control calves, the IL-8 and TNF-α levels in blood were significantly increased at week 6 compared to week 4, while IL-6 and IL-1β concentrations were slightly reduced (**Figure 7K-N**). Intranasal GOS significantly decreased the blood concentrations of IL-6 and IL-1β at week 4, but not at week 6, while no effects on IL-8 and TNF-α levels were observed (**Figure 7K-N**).

The correlations were analyzed to investigate the relation between neutrophils and cytokines in BALF of control and GOS-spray groups at week 5. Significant positive correlations were measured between the number of neutrophils and the concentrations of TNF-α in BALF of control (Chapter 3, **Figure 4C**) and GOS-spray (**Figure S3G**) groups at week 5.

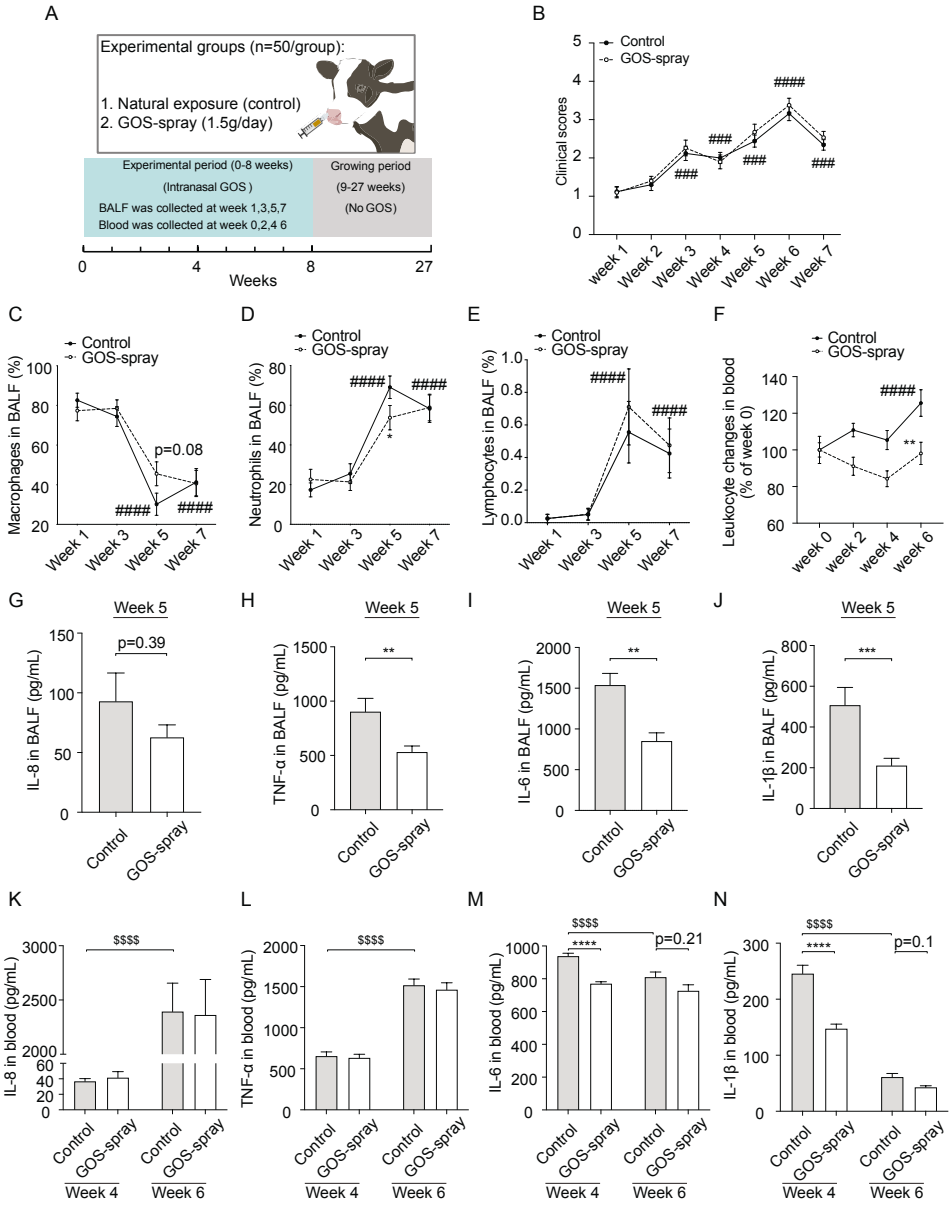


Figure 7. Effect of intranasal GOS on the clinical scores and the cell composition and cytokine/chemokine levels in BALF and blood. (**A**) Timeline and design of the experiment. Calves (n=100, 50 calves/group) were naturally exposed to respiratory pathogens in the environment and treated with or without intranasal GOS (GOS-spray) for 8 weeks during early life followed by 19 weeks (week 9-27) without GOS administration. BALF and blood were collected at week 1, 3, 5, 7 and week 0, 2, 4, 6, respectively. (**B**) Clinical scores were evaluated over time for all calves (n=100, 50 calves/group). (**C**-**E**) Percentage of macrophages, neutrophils, and lymphocytes in total BALF cells was measured at week 1, 3, 5 and 7 for a subset of calves (n=40, 20 calves/group). (**F**) Leukocyte changes in blood were determined at week 0, 2, 4 and 6 for the same subset of calves (n=40, 20 calves/group). (**G**-**J**) IL-8, TNF-α, IL-6, and IL-1β levels in BALF were measured by ELISA at week 5 for the same subset of calves (n=40, 20 calves/group). (**K**-**N**) IL-8, TNF-α, IL-6, and IL-1β levels in blood were determined by ELISA at week 4 and 6 for the same subset of calves (n=40, 20 calves/group). **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 (GOS-spray vs control at that timepoint); ###*P*<0.001; ####*P*<0.0001 (control week 3-7 vs week 0 or 1); $$$$*P*<0.0001 (control week 6 vs week 4). Data are presented as means ± SEM.

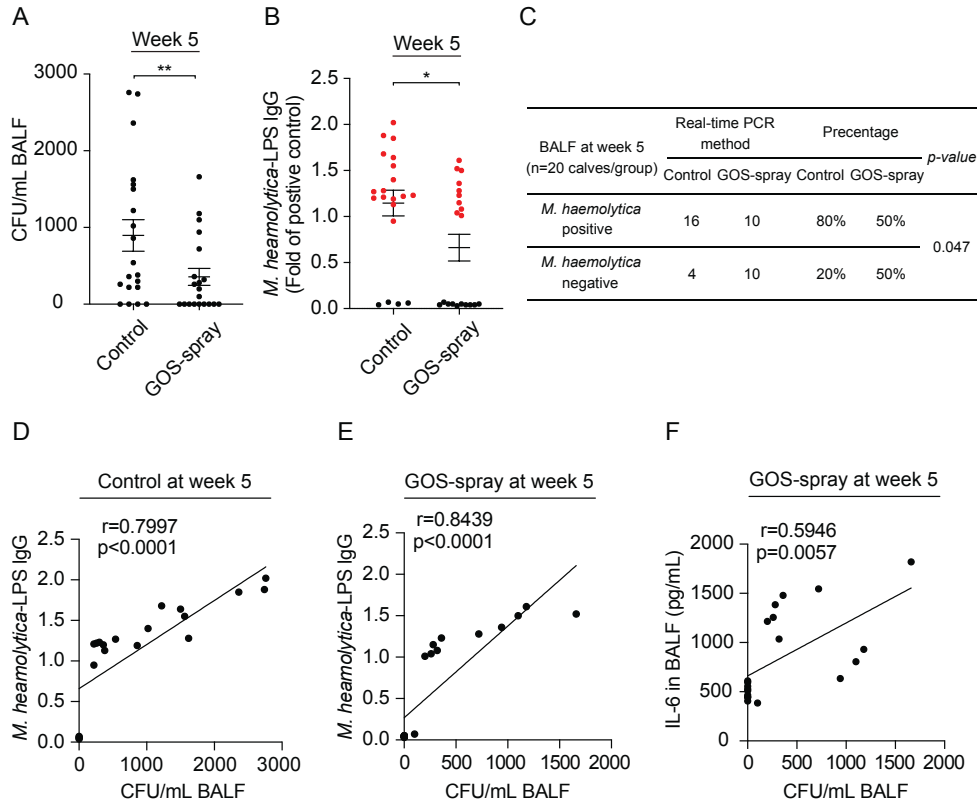
Intranasal application of GOS reduces the positivity of *M. haemolytica* in the lungs

Colonization and invasion of *Pasteurellaceae* (e.g., *M. haemolytica*) have been implicated as an important prerequisite for bovine lung infections [18]. Here, the number of *Pasteurellaceae* in the lungs was determined by swabbing BALF on the 5% sheep blood agar plate and counting CFUs [3]. *Pasteurellaceae* in BALF increased significantly over time and peak amount was reached during week 5 (**Figure S3H**). Compared with the control group, the *Pasteurellaceae* CFUs in BALF were significantly decreased by intranasal GOS at week 5 and maintained at a lower level over time (**Figure 8A** and **Figure S3H**).

M. haemolytica is one of the main *Pasteurellaceae* that contribute to the development of bovine lung infections. It releases LPS to produce pro-inflammatory cytokines/chemokines and promote the lung lesions through the stimulation of airway epithelial cells and leukocytes [5]. Here, the *M. haemolytica*-LPS IgG levels was detected in BALF at week 5 (the same timepoint for the measurements of cytokines/chemokines in BALF). The number of calves positive for *M. haemolytica*-LPS IgG (red dots) within the control group was 80% (16/20) at week 5 (**Figure 8B**). Intranasal GOS significantly reduced the *M. haemolytica*-LPS IgG levels in BALF at week 5 (**Figure 8B**).

The detection of *M. haemolytica*-LPS IgG levels is an indirect method for identifying *M. haemolytica* in BALF. Hence, the positivity for *M. haemolytica* was detected by real-time PCR in BALF of all calves at week 5. **Figure 8C** showed that 80% (16/20) of control calves were positive for *M. haemolytica*. In line with the data from *M. haemolytica*-specific LPS in BALF (**Figure 8B**), this indicated that *M. haemolytica* might be one of the pathogens associated with the lung infection of calves at week 5. Intranasal GOS showed a reduction in the number of calves positive for *M. haemolytica* (50% vs 80%, p=0.047) (**Figure 8C**).

The correlations were analyzed to investigate the relation between the CFUs of *Pasteurellaceae* and the levels of *M. haemolytica*-LPS IgG and cytokines in BALF at week 5. Significant positive correlations were observed between the *Pasteurellaceae* CFUs and the *M. haemolytica*-LPS IgG levels in BALF of both control and GOS-spray groups at week 5 (**Figure 9D** and **E**). In addition, positive correlations were observed between the *Pasteurellaceae* CFUs and the IL-6 concentrations in BALF of GOS-spray group at week 5 (**Figure 9F**).



BALF at week 5 (n=20 calves/group)	Real-time PCR method		Precentage		*p-value*
	Control	GOS-spray	Control	GOS-spray	
M. haemolytica positive	16	10	80%	50%	0.047
M. haemolytica negative	4	10	20%	50%	

Figure 8. Intranasal GOS reduces the positivity of *M. haemolytica* in the lungs. (**A**) BALF from week 5 was inoculated onto the 5% sheep blood agar plates and CFUs of

Pasteurellaceae were counted for the same subset of calves (n=40, 20 calves/group). (**B**) *M. haemolytica*-LPS IgG levels were detected in BALF of the same subset of calves at week 5 by ELISA method (n=40, 20 calves/group). Compared to the positive control, fold changes were calculated. Black dots represent *M. haemolytica*-LPS IgG negative calves. Red dots represent *M. haemolytica*-LPS IgG positive calves. (**C**) Number and percentage of *M. haemolytica* positive calves according to the presence in BALF by real-time PCR method. (**D**-**E**) Correlations between the CFUs of *Pasteurellaceae* and the levels of *M. haemolytica*-LPS IgG in BALF of control and GOS-spray groups at week 5. (**F**) Correlations between the CFUs of *Pasteurellaceae* and the concentrations of IL-6 in BALF of GOS-spray group at week 5. **P*<0.05; ***P*<0.01 (GOS-spray vs control group). Data are presented as means ± SEM.

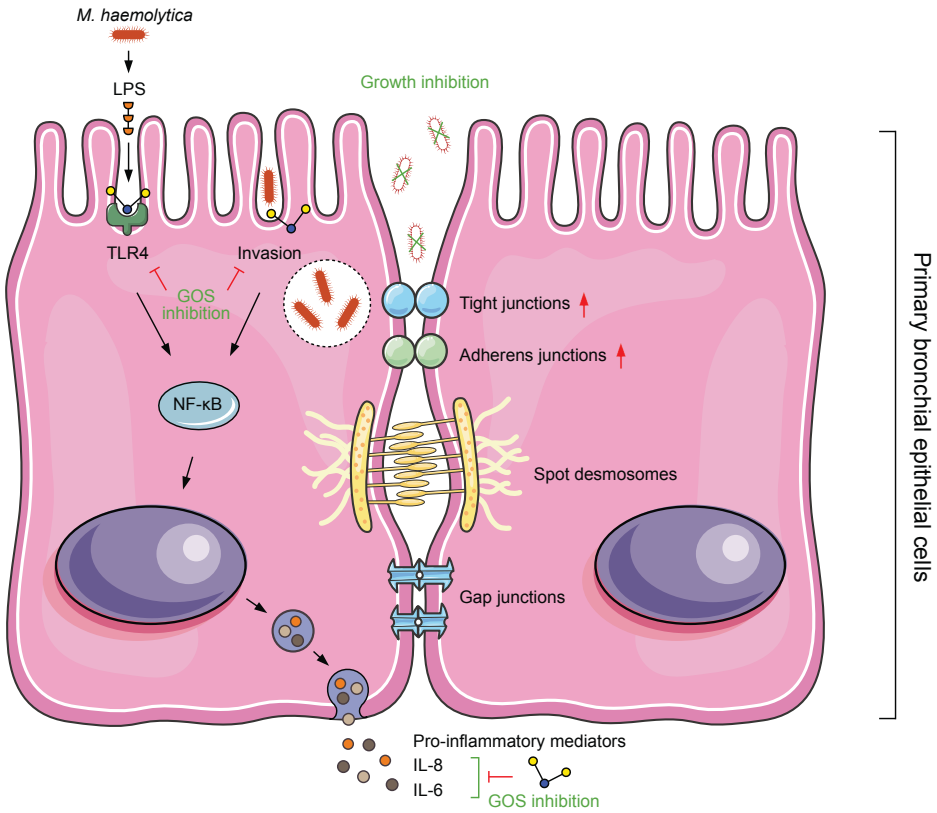


Figure 9. A possible mechanism for GOS to protect the bronchial epithelial cells from *M. haemolytica*-induced infection and subsequent inflammation. On the one hand, GOS may lower *M. haemolytica* viability by damaging the bacterial cell membrane. On the other hand, GOS is thought to act as a soluble decoy receptor, resulting in reduced adhesion to and invasion of airway epithelial cells by *M. haemolytica*. This inhibits the release of cytokines/chemokines and prevents *M. haemolytica* from entering the cells through endocytosis and damaging the airway barrier integrity. In addition, GOS can also inhibit the release of cytokines/chemokines induced by one of the virulence factors of *M. haemolytica*, LPS, thereby protecting the impairment of barrier integrity caused by the accumulation of inflammatory mediators.

Discussion

In this study, we described for the first time that intranasal (local) administration of GOS via spray relieved respiratory inflammation/infection, which might be due to their interesting anti-bacterial, anti-adhesion and anti-inflammatory properties. Airway epithelial cells are the first line of defense against pathogens from invading and infecting lungs [6], and inhibition of bacterial attachment to epithelial cells by carbohydrates has been known for more than three decades [19, 20].

As the outbreaks of fatal airway infections by *M. haemolytica* in calves have become increasingly apparent in recent years [2, 4], this respiratory pathogen was used for our *in vitro* studies. In the present study, we reported for the first time that GOS has the capacity to reduce bacterial viability *in vitro*. GOS are commonly supplemented to infant formula to simulate the effects of HMOs [13]. Lars Bode *et al.* showed that non-sialylated HMOs directly and specifically inhibited the growth of GBS. The bacterial glycosyltransferase encoded by the gbs0738 gene might catalyze the incorporation of these HMOs into the bacterial cell wall to inhibit the growth of GBS [14]. HMOs were able to increase the permeability of GBS membranes in a concentration-dependent manner to exert anti-bacterial activity [21, 22], which is in agreement with our findings. Moreover, increased membrane permeability also contributes to the improvement of antibiotic efficacy [23]. In the present study, the live/dead ratio of *M. haemolytica* at 24h was around 3.5, indicating 22% of *M. haemolytica* might be dead. This can be due to nutritional competition, the 646-fold proliferation of *M. haemolytica* from 0h (0.82×10^5 CFU/ml) to 24h (0.53×10^8 CFU/ml) increased the depletion of nutrients. NDOs are considered to be nutrients that promote the growth of commensal bacteria (e.g., *Bifidobacterium* and/or *Lactobacillus* species) [13]. In Chapter 6, FOS ≥1% promoted the growth of *M. haemolytica in vitro*. However, in the present study, GOS did not seem to be used as a nutrient for *M. haemolytica*. Therefore, the inhibition of *M. haemolytica* viability might be specific for GOS.

Chitosan oligosaccharide (COS) displayed inhibitory effects on bacterial growth due to increased pore formation and permeabilization of the cell wall of *Bacillus cereus*, whereas the blockage of nutrient flow caused by aggregation of COS is the main reason for the growth inhibition and lysis of *Escherichia coli* [24]. Here, lower concentrations of GOS (4%) showed growth inhibition of *M. haemolytica*, while higher concentrations of GOS (8% and 16%) strongly inhibited the viability of *M. haemolytica*, which may explain the inhibited bacterial amount and *M. haemolytica* positivity in BALF induced by intranasal GOS. GOS might reduce proliferation or the number of *M. haemolytica* in the upper respiratory tract,

limiting translocation of the pathogen to the lungs. Although more than 4% GOS showed toxicity to PBECs after 48h incubation, the presence of bronchial mucus in calves may dilute the intranasal GOS solution [25]. Moreover, mucociliary clearance, will most probably quickly eliminate intranasal GOS from the airways, as observed in our pilot experiment. In addition, compared with the monolayer model, the calf airways are populated by a diverse microbiota. This commensal microbiota directly inhibits the growth of bacterial pathogens, likely through the use of available nutrients (e.g., GOS) [26].

In order to cause a lung infection, *M. haemolytica* has developed various strategies for crossing the airway barrier, including adhesion and entry into cells, or simply killing cells to disrupt the airway barrier [7, 15], characterized by destruction of tight and adherens junctions and release of proinflammatory cytokines/chemokines by bronchial epithelial cells [7]. To gain more insights into the anti-inflammatory effects of GOS, an *in vitro* infection model using PBECs was established [7]. Interestingly, we observed that 2% GOS inhibited the invasion of and adhesion to PBECs by *M. haemolytica* without affecting the bacterial growth in the supernatants. This might be due to the small amount of *M. haemolytica* that invades PBECs, while a significant amount is exponentially growing in the supernatant and affecting PBECs by releasing virulence factors (e.g., LPS). GOS have been reported as anti-adhesives and decoy glycan receptors, which is mainly due to the structural similarity between GOS and various cell surface glycans [13]. GOS competitively inhibit the adhesion of enteropathogenic *E. coli* [27] and *Cronobacter sakazakii* [28] to host epithelial cells and the binding of *Vibrio cholerae* toxin to the toxin receptor (GM-1) of host cells [29]. In the present study, although 2% GOS does not have the ability to inhibit the growth of *M. haemolytica*, it might act as a decoy glycan to competitively bind to the carbohydrate moiety on bronchial epithelial cells, preventing the invasion and adhesion of *M. haemolytica*.

In our study, GOS induced a decrease in the release of cytokines and chemokines in PBECs after *M. haemolytica*/LPS exposure, and in BALF and blood of calves after natural exposure, which is consistent with previous *in vitro* and *in vivo* studies related to the anti-inflammatory activity of GOS in human Caco-2 cell line and murine models [30, 31]. In addition, GOS prevented the TEER decrease and altered expression of tight and adherens junction-related proteins (ZO-1 and E-cadherin) induced by *M. haemolytica*, maintaining the functional airway epithelial barrier integrity. On one hand, the inhibition of 2% GOS on the adhesion to and invasion of PBECs by *M. haemolytica* might prevent the subsequent release of inflammatory mediators (e.g., cytokines and chemokines) and the disruption of the airway epithelial barrier (**Figure 9**). On the

other hand, GOS might eliminate the adverse effects of the released endotoxins (e.g., LPS) and exotoxins (e.g., leukotoxin) (from *M. haemolytica)* on bronchial epithelial cells, due to their ability to interfere or neutralize bacterial toxins [13]. Although in our study undifferentiated PBECs were used, the liquid-liquid system does not force the maturation and ciliation of epithelial cells and can retain some epithelial progenitor cells and non-ciliated cells, which are also present/play an important role in the airways in health and disease [32, 33]. In addition, our *ex vivo* model can create an intact airway epithelial barrier with well-developed junctional complexes and exhibit an inflammatory response after stimulation with *M. haemolytica* and other stimulants (LPS and flagellin).

Remarkably, in addition to the invasion of PBECs by *M. haemolytica*, virulence factors released by *M. haemolytica* (e.g., LPS) also have the ability to induce airway epithelial inflammation and barrier dysfunction, possibly through the recognition of TLRs [2, 7]. In our study, the TLR4 protein expression and downstream p-p65 were increased in PBECs after *M. haemolytica* exposure, indicating activation of TLR4/NF-κB pathway, resulting in the induction of IL-8 and IL-6 [34]. Furthermore, the TLR4 inhibitor, CLI-095, decreased the TLR4 protein expression, the NF-κB p65 phosphorylation, and the release of IL-8 and IL-6 in PBECs after *M. haemolytica* exposure, suggesting a potential role for TLR4 in the *M. haemolytica*-induced inflammation [34]. However, CLI-095 cannot prevent the *M. haemolytica*-induced disruption of the PBEC monolayer integrity. This might be explained by the ability of *M. haemolytica* to destroy the epithelial barrier mainly through adhesion, invasion, and explosive replication [15] instead of activation of TLR4. Interestingly, GOS (2%) can not only prevent the adhesion and invasion of *M. haemolytica* to PBECs but also inhibit the *M. haemolytica*-induced activation of the TLR4/NF-κB pathway (**Figure 9**). This might be the reason that although both GOS and CLI-095 inhibit TLR4/NF-κB pathway, the anti-inflammatory effect of GOS seems to be better than that of CLI-095. In addition, GOS also decreased the LPS- but not flagellin-induced epithelial inflammation in both human and bovine airway epithelial cells. Although these results provide indirect evidence, it can be suggested that GOS might specifically inhibit the recognition of LPS by TLR4. Increasing evidence showed that NDOs are directly or indirectly involved in the TLR4/NF-kB pathway [13, 35, 36]. For example, HMOs (2'-fucosyllactose and 6'-sialyllactose) were reported to inhibit TLR4 expression and signaling in mouse and piglet necrotizing colitis models and human (infant) intestinal explants, probably due to the capacity of HMOs to directly dock into the LPS-binding pocket of TLR4 [37].

Intranasal GOS were innovatively applied to calves with naturally occurring lung infections due to the benefits of GOS described *in vitro*. Breastfeeding

infants ingest mother milk several times per day, bathing the nasopharynx for several minutes at each feeding with a solution high in HMOs, which might inhibit local bacterial adherence [20]. *In vivo,* it has been observed that oral supplemented GOS and/or FOS can also have beneficial effects on the airways [31, 38]. Our data showed that intranasal GOS partly reduced lung inflammation, indicated by a decreased concentrations of TNF-α, IL-6 and IL-1β in BALF and IL-6 and IL-1β in blood. Although the total cell numbers (mainly pulmonary leukocytes) in BALF did not reduce in the GOS-spray group, intranasal GOS restored markedly the ratios of macrophages to neutrophils, as indicated by more macrophages and fewer neutrophils, which is commonly observed in the lower respiratory tract of healthy calves [17, 39]. Neutrophils can be recruited and activated by multiple pro-inflammatory mediators including IL-8, TNF-α, IL-6 and IL-1β [40, 41], while excessive accumulation of neutrophils and pro-inflammatory mediators can mediate tissue damage in the lungs [42].

GOS also seemed to reduce the number of leukocytes in blood. The differences between control and GOS-spray groups were more obvious in parameters measured in BALF compared to blood, which might be related to the local GOS administration and possible rapid removal from the distal trachea by mucociliary clearance [19, 43]. Furthermore, it cannot be excluded that part of the effects of GOS might be related to spillover in the esophagus. In addition, the anti-inflammatory and immunomodulatory effects of GOS occurred during experimental week 5 and seemed to disappear during week 7, which might be related to (1) the group antibiotic treatments based on clinical scores at week 6 and/or (2) the innate immune system activation (increased BALF neutrophils and blood leukocytes) during week 5/6, which contributes to partly eliminating/phagocytosing the pathogens in the lungs.

It has been described that inoculation of BALF on sheep blood agar is a fast and economical method for screening *Pasteurellaceae* (*M. haemolytica, P. multocida and H. somni*), with usually pure cultures, high isolation rates and less nasopharyngeal contamination [3, 44-46]. Our study showed local administration of GOS suppressed the *Pasteurellaceae* number in BALF, however in this study we cannot distinguish between *M. haemolytica* and other *Pasteurellaceae* (*P. multocida and H. somni*) on sheep blood agar. Therefore, *M. haemolytica*-LPS IgG detection and real-time PCR were used to determine the positive for *M. haemolytica* in BALF. Up to 80% of calves are positive for *M. haemolytica* in BALF, indicating that *M. haemolytica* might be one of the pathogens involved in the lung infections of present study. Interestingly, intranasal GOS reduced the *M. haemolytica*-LPS IgG levels and *M. haemolytica* positivity in BALF/lungs. Furthermore, positive correlations were observed between the *Pasteurellaceae*

CFUs and *M. haemolytica*-LPS IgG levels in BALF of GOS-spray group, indicating a reduced number of *M. haemolytica* may exist in the lower respiratory tract of calves treated with intranasal GOS, which might be due to the anti-bacterial and anti-invasive effects of GOS. In addition, the suppressed *Pasteurellaceae* (possibly *M. haemolytica*) number by GOS-spray may contribute to the reduced cytokine concentrations and neutrophil numbers in BALF. The release of *M. haemolytica*-LPS is one of the causes of airway inflammation observed in lung infections [5, 18]. The decreased airway inflammation in infections might be related to the specific inhibition of LPS-induced inflammation by GOS.

In addition, the changes of nasal commensal bacteria may contribute to the *M. haemolytica* inhibition in BALF. GOS intervention has been widely used to stimulate the growth of *Bifidobacterium* and/or *Lactobacillus* species in the gut [13]. A recent study in calves showed that intranasal administration of *Lactobacillus* strains reduces the nasal colonization of *M. haemolytica*, which might be due to the competition for adherence to the mucosa or direct growth inhibition [46].

Single-pathogen challenge models experimentally inoculate a large number of pathogenic bacteria (e.g., *M. haemolytica*) in the lower respiratory tract [46, 47], however, ignore the complexity of the infection, involving the invasion of opportunistic bacteria caused by stress, weakened immunity, environmental factors, close contact transmission, etc. [5]. During week 5 of our natural exposure model, the clinical score increased 1.5-fold, macrophages decreased extensively, lymphocytes increased slightly, while neutrophils increased expansively (11-fold) in BALF and were the predominant cell type in BALF, indicating the presence of lung infections, most probably bacterial infections [17, 45], are present from week 5. Therefore, chemokine/cytokine parameters in BALF were mainly measured at week 5. In addition, the peak number of *Pasteurellaceae* in BALF also supports our conclusion that a bacterial outbreak was present in the lungs during week 5. Blood parameters were measured one week prior to or one week after BALF collection, to minimize the disadvantages of invasive measurements of both BALF and blood collection during the same week [5]. However, in naturally occurring or experimentally inoculated lung infections, clinically healthy or asymptomatic individuals might be present [3, 46], which is similar to the pattern of human respiratory infections. This could be the reason why the effect of GOS-spray on BALF composition and clinical scores might be underestimated. In addition, the application of antibiotic strategy against lung infections for all groups may (partly) reduce the severity of lung infections which could interfere with obtained results. Furthermore, the insensitivity of

clinical scores to the diagnosis of (subclinical) lung infections may also lead to contrasting results as compared to the measurements of cell composition and cytokine/chemokine levels in BALF/blood [45].

Conclusions

In summary, it can be concluded that 8% and 16% GOS significantly reduced the viability of *M. haemolytica in vitro,* while 4% GOS mainly lowered the growth of *M. haemolytica*. Next to that, low concentrations of GOS (2%) prevent *M. haemolytica* from adhering to and invading of PBECs and inhibit the activation of the TLR4/NF-κB pathway, which contributes to a reduced cytokine/chemokine release and restored airway epithelial barrier. In addition, although it has no effects on clinical scores, the intranasal administration of GOS can relieve the inflammatory response and reduce the *M. haemolytica* positivity during naturally occurring lung infections in calves. Therefore, this study proposes a promising new strategy for the clinical evaluation of local application of GOS in other animals and humans (children) suffering from respiratory infections.

Materials and Methods

Quantification of *M. haemolytica* growth

M. haemolytica isolated from infected lungs of a pneumonic calf was cultured on blood agar plate enriched with 5% sheep blood (bioTRADING, Mijdrecht, The Netherlands) at 37°C.

For bacterial quantification, *M. haemolytica* (1 x 10^5 CFU/mL) was incubated in 96-well plates with or without increasing concentrations of GOS (0.25%, 0.5%, 1%, 2%, 4%, 8% or 16%) for 0, 1, 3, 6, 12 or 24h. After incubation, *M. haemolytica* growth was determined by measuring the turbidity of supernatants at OD600nm using a microplate reader (Promega Corp., Madison, WI). The minimum inhibitory concentration was recorded as the lowest concentration of GOS at which no bacterial growth was observed.

Bacterial membrane permeability assay

M. haemolytica (1 x 10^5 CFU/mL) was incubated in 96-well plates with or without different concentrations of GOS (0.5%, 1%, 2%, 4%, 8% or 16%) for 0h and 24h.

After incubation, a LIVE/DEAD BacLight assay (Invitrogen, Thermo Fisher Scientific, Waltham, MA) was applied to assess bacterial cell membrane permeability according to manufacturer's instructions. Briefly, *M. haemolytica* was stained with propidium iodide (PI) and SYTO 9 dye for 15 min prior to evaluation with a microplate reader (Promega Corp.) at excitation/emission 485/530nm (green, SYTO 9) and 485/630nm (red, PI). SYTO 9 is a dye that passes through intact membranes to stain live cells green, while PI is a larger molecular dye that can only pass-through membranes that have breached integrity to stain dead cells red [21]. The live/dead ratio was calculated as a ratio of green to red fluorescence (ratio=green/red). The percentage of *M. haemolytica* with impaired membrane permeability was calculated as ratio%=red/(green+red) x 100%.

Minimum bactericidal concentration assay

M. haemolytica (1 x 10^5 CFU/mL) was cultured in 96-well plates with or without different concentrations of GOS (4%, 8% or 16%) for 0h and 24h. Then, the supernatants were diluted and sub-cultured onto 5% sheep blood agar plates overnight at 37°C. *M. haemolytica* CFUs on each agar plate were counted and calculated as CFU/mL based on the dilution factor of 100 for *M. haemolytica* group at 0h, dilution factor of 50,000 for *M. haemolytica* group and 4% GOS treatment at 24h, and dilution factor of 100 for 8% and 16% treatments at 24h. When the counted *M. haemolytica* CFUs were lower than the initial CFUs (1 x 10^5 CFU/mL), the surviving *M. haemolytica* was calculated and represented as the percentage relative to the initial *M. haemolytica*.

Isolation and culture of PBECs

Isolation and culture of PBECs were conducted as previously described [7]. Briefly, PBECs were isolated from bovine bronchial epithelium obtained from the lungs of freshly slaughtered calves aged 6-8 months provided by Ekro bv (Apeldoorn, The Netherlands). After digesting of the bronchial epithelium, PBECs were collected and grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and attached to collagen-coated plates in serum-free RPMI-1640 medium for 2-3 days until reaching near-confluence (70-90%) and then replaced with RPMI-1640 medium containing 10% FBS for future culture and experiments as described before [7].

Human alveolar epithelial cell line culture

Human Type II alveolar epithelial cells (A549; ATCC, Manassas, VA) were grown in Ham's F-12K Medium (Gibco, Thermo Fisher Scientific) supplemented with 10% FBS (Sigma-Aldrich, Zwijndrecht, The Netherlands), penicillin (100 U/mL, Sigma-Aldrich) and streptomycin (100 µg/mL, Sigma-Aldrich) in 5% CO_2 at 37°C.

PBEC and A549 treatments

PBECs were cultured at a density of 1 x 10^6 cells/mL in 96- or 6-well plates pre-coated with collagen, fibronectin and BSA as previously described [7]. After reaching near-confluence, these PBECs were pretreated with different concentrations of GOS for 24h or CLI-095 (1 μM, Toll-like receptor 4 inhibitor, Invivogen, San Diego, CA) for 3h prior to 24h *M. haemolytica* (1 x 10^5 CFU/mL), LPS (10 μg/mL; isolated from *E. coli* O111:B4, Sigma-Aldrich) or flagellin (10 ng/mL; isolated from *P. aeruginosa*, Invivogen) stimulation. After stimulation, PBECs were harvested, and supernatants were collected and stored at -20 °C until analysis.

A549 cells were cultured at a density of 0.5 x 10^5 cells/mL in 24 or 6-well plates. After reaching near-confluence, A549 cells were pretreated with different concentrations of GOS for 24h prior to 24h LPS (10 μg/mL; isolated from *E. coli* O111:B4, Sigma-Aldrich) or flagellin (10 ng/mL; isolated from *P. aeruginosa*, Invivogen) stimulation. After stimulation, supernatants were collected and stored at -20 °C until analysis.

TEER measurement and paracellular tracer flux assay

PBECs (1 x 10^6 cells/mL, 300 μL) were added to the apical compartment of the permeable 0.3 cm^2 high pore density polyethylene membrane transwell inserts (Corning, NY) placed in a 24-well plate and 700 μL cell culture medium (RPMI-1640 containing 10% FBS) was added to the basolateral compartment, prior to incubation at 37°C in a humidified atmosphere of 95% air and 5% CO_2. TEER of PBECs was measured by a Millicell-ERS Volt-Ohm meter (Millipore, Merck, Darmstadt, Germany) every 2 days. The culture medium from the basolateral and apical compartment was refreshed after TEER measurement and experiments started at day 11 when sustained TEER values around 600 Ω·cm^2 were reached as we described before [7].

PBECs were incubated with *M. haemolytica* (1 x 10^5 CFU/mL) from the apical side with or without 24h pretreatment with GOS or 3h pretreatment with CLI-095 at both the apical and basolateral compartments. TEER was measured at 0 and 24h after *M. haemolytica* exposure. Thereafter, a membrane-impermeable molecule, lucifer yellow (molecular mass of 0.457 kDa, 20 μg/mL; Sigma-Aldrich), was added to the apical compartment for 4 h, and the paracellular flux was determined by measuring the fluorescence intensity at the basolateral compartment with a fluorometer (Promega Corp.) set at excitation/emission wavelengths of 410/520nm. After measurement, PBECs were harvested for immunofluorescence staining and western blotting, and supernatants were collected and stored at -20 °C until analysis.

LDH assay

PBECs or A549 cells were grown in 96-well plates. After the *M. haemolytica*, LPS or flagellin stimulation with or without GOS pretreatment, supernatants were collected, and the cytotoxic effect was evaluated by measuring LDH leakage. LDH was measured in the supernatants using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega Corp.) according to manufacturer's instructions.

Thiazolyl blue tetrazolium bromide (MTT) assay

PBECs or A549 cells were grown in 96-well plates and pretreated with or without GOS following by *M. haemolytica*, LPS or flagellin stimulation and the viability of PBECs or A549 cells was measured using MTT assay. MTT (Sigma-Aldrich) was dissolved at a final concentration of 0.5 mg/mL in culture medium. Each culture well was delicately washed with pre-warmed (37°C) PBS before adding a 120 µL MTT solution. After 3h of incubation (37°C, 5% CO_2), the formed formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) and absorbance was read at 595nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA).

Bacterial invasion and adhesion assay

PBECs were grown in 6-well plates. After the different treatments (see above), supernatants were collected, and absorbance measured at 600nm using a microplate reader (Promega Corp.). *M. haemolytica* growth in the supernatants was presented as a percentage of control.

To quantify the number of invaded bacteria, extracellular *M. haemolytica* was killed by adding fresh medium containing 250 µg/mL gentamicin (Sigma-Aldrich) to PBECs for 3h incubation. PBECs were rinsed three times with 37°C PBS and lysed by 1% Triton X-100. The suspensions were collected, diluted, and sub-cultured overnight at 37°C on 5% sheep blood agar plates. *M. haemolytica* numbers were determined by counting *M. haemolytica* CFUs on each agar plate and calculated as CFU/mL based on the dilution factor of 1,000.

To calculate the number of adherent bacteria, PBECs were rinsed three times with 37°C PBS to remove non-adherent *M. haemolytica* and lysed by 1% Triton X-100. The suspensions were collected, diluted, and sub-cultured onto 5% sheep blood agar overnight at 37°C. *M. haemolytica* numbers were determined by counting *M. haemolytica* CFUs on each agar plate and calculated as CFU/mL based on the dilution factor of 1,000. Adherent bacteria were calculated as: [the number of total bacteria] – [the number of invaded bacteria].

Western blotting

Cell lysates of PBECs after different treatments were prepared by adding RIPA cell lysis buffer (Thermo Fisher Scientific) containing protease inhibitors (Roche Applied Science, Pennsburg, Germany). Total protein content was estimated by bicinchoninic acid analysis (Pierce, Thermo Fisher Scientific) according to the manufacturer's protocol. Samples were loaded onto polyacrylamide gradient gels (4-20% Tris-HCl, Bio-Rad Laboratories) and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). The membranes were blocked with PBS containing 0.05% Tween-20 (PBST) and 5% milk proteins for 1h at room temperature and incubated with primary antibodies at 4 °C overnight (ZO-1, 1:1,000, Abcam, Cambridge, UK; E-cadherin, 1:1,000, BD Biosciences, San Jose, CA; TLR4, 1:1,000, Thermo Fisher Scientific; p-p65, 1:1,000 and β-actin, 1:5,000, Cell Signaling Technology, Beverly, MA) followed by washing blots in PBST. Appropriate horseradish peroxidase-coupled secondary antibodies from Dako (Agilent Technologies, Santa Clara, CA) were applied for 1h. Membranes were incubated with ECL western blotting substrates (Bio-Rad Laboratories) prior to obtaining the digital images. Digital images were acquired with the Molecular Imager (Gel Doc XR System, Bio-Rad Laboratories) and analyzed with Image lab 5.0 (Bio-Rad Laboratories).

Immunofluorescence

The tight junction and adherens junction proteins of PBECs were detected using immunofluorescence microscopy. PBECs were fixed with 10% formalin (Baker, Deventer, The Netherlands) and after washing with PBS, the cells were permeabilized with PBS containing 0.1% (v/v) Triton-X-100 for 5 minutes, followed by blocking with 5% serum in 1% (w/v) BSA/PBS for 30 minutes at room temperature. Thereafter, PBECs were incubated overnight with primary antibodies ZO-1 (1:50, Abcam) and E-cadherin (1:50, BD Biosciences) followed by incubation with Alexa-Fluor conjugated secondary antibodies (Invitrogen, Thermo Fisher Scientific) for 1h at room temperature in dark. Nuclear counterstaining was performed with DAPI containing anti-fade reagent (ready to use, Invitrogen). ZO-1 and E-cadherin were visualized, and images were taken using the Keyence BZ-9000 (KEYENCE Corporation, Itasca, IL). Fluorescence intensity was quantified by Image J 1.8.0, (National Institutes of Health, Bethesda, MD) and represented as fluorescence intensity (vs DAPI).

Animal experiment design

The animal experiment was conducted at the research facilities of the VanDrie Group (Scherpenzeel, The Netherlands) and approved by the Animal Welfare Body of Wageningen University (2017.W-0017.001, Wageningen, The Netherlands) and the Dutch Central Authority for Scientific Procedures on

Animals (project AVD1040020185828).

The experiment consisted of 2 periods, an experimental period, and a growing period. Period 1 (experimental period) started when calves arrived at the experimental facilities (~18 days of age) and lasted from experimental week 1 to 8 in which GOS were intranasally applied via spray once per day and most of the individual measurements were conducted during this period. Period 2 lasted from experimental week 9 to slaughter at experimental week 27. In period 2, no oligosaccharide treatments were applied, and all calves received the same diet. Measurements and analyses were performed for all calves or for a subset of calves. The subset of calves included 2 calves per pen and 20 calves per group and was selected on body weight at arrival, closest to the average body weight of all calves at arrival.

During these periods, all calves were naturally exposed to pathogens in the environment. Individual antibiotic treatment was applied when required based on clinical signs of illness as assessed by the animal caretakers. The number of applied individual antibiotic treatments did not differ between the control and GOS-spray groups ($P > 0.1$). Group antibiotic treatment was applied equally to all groups if 10% of the calves had been treated within 5 days, or if 5% of the calves had become ill within 24h or when the situation required group antibiotics in the expert judgement of a veterinarian.

The *in vivo* study described in this article was part of a large calf trial, including 300 calves randomly assigned to a control group, a GOS-spray group and four groups with different dietary interventions. In accordance with the purpose of this study, investigating the effect of intranasal GOS on lung infection, we reported the results of the analyses of the control and GOS-spray groups (100 of 300 calves).

Animals, housing and feeding

Calves were housed in a mechanically ventilated stable throughout the experiment. The ambient lighting consisted of natural lighting plus artificial lighting from 0600 to 1800 h. Calves were housed in pens (9 m^2) containing wooden-slatted floors. In the first 6 weeks after arrival, individual housing was applied (1.2 m^2/calf) by placing stainless steel fences within the pens. After 6 weeks, the individual fencing was removed, and calves were housed in groups of 5.

In period 1, 100 male Holstein Friesian calves (43.3 ± 0.32 kg, means ± SEM) of German origin were used and assigned randomly to 2 groups. Group 1 as a control group included 50 calves. Group 2 included 50 calves that received intranasal GOS (10 mL warm saline/1.5 g GOS, Vivinal GOS syrup,

FrieslandCampina, Amersfoort, The Netherlands). GOS were dissolved in warm saline and sprayed into the nasopharynx through an intranasal applicator (Rispoval, Zoetis B.V., The Netherlands) mounted on the top of the syringe.

All calves received milk replacer (MR) twice a day in the diet. The MR mainly contained 527 g/kg whey powder, 35 g/kg lactose, 52 g/kg delactosed whey powder, 50 g/kg whey protein concentrate, 60 g/kg soy protein concentrate, 50 g/kg soluble wheat protein, 3 g/kg pea fiber, 179.4 g/kg fat sources, 9.7 g/kg calcium formate, 2 g/kg citric acid, 4 g/kg sodium bicarbonate, 3.5 g/kg mono ammonium phosphate, 9.8 g/kg lysine, 2.4 g/kg methionine, 1.3 g/kg threonine, 0.2g/kg aroma and 10 g/kg premix.

Blood sampling and hematological analyses

Blood samples were collected of all calves by venipuncture of the jugular vein at arrival before the first MR feeding (baseline, week 0), and additionally at experimental week 2, 4 and 6 from 20 calves per group. Blood was collected in 9 mL and 4 mL K_2-EDTA tubes and was kept on ice for collection of plasma or kept at room temperature for analysis of leukocyte numbers the same day by fluorescence flow cytometry using a Sysmex 1800iV (Sysmex Europe GmbH, Norderstedt, Germany), respectively. Plasma was collected after centrifugation at 2,000 x *g* and 4°C for 20 min and was stored at -20°C pending further cytokine/chemokine measurement by ELISA.

Bronchoalveolar lavage fluid (BALF) sampling and phenotyping

BALF samples were obtained by use of a technique adapted from Caldow *et al*. [48]. Briefly, a calf was restrained in the feeding fence and the head of calf was lifted and extended so that the nasal bone was parallel to the ground. Ethanol (70%) was used to clean the nose/nostrils of the calf. A sterilized 100 cm BAL catheter was then inserted through a naris and blindly guided through the nasal passage into the trachea until the end was wedged in a bronchus. The correct placement of the catheter was verified by elicitation of the coughing reflex, the outstretch of the tongue, movement of air into and out of the catheter with each breath, and the absence of rumen contents, odor, and gurgling from the catheter. Once wedged in the appropriate location, a syringe was connected to the catheter and a total of 30 mL of sterile saline (0.9% NaCl, 37°C) solution was infused into the tube and fluid was immediately aspirated from the bronchus. BALF (18.5 ± 0.39 mL, means ± SEM) was obtained from each calf and was stored in a 50 mL tube on ice until further processing in the lab the same day.

Thereafter, BALF was filtered by passing through a 70 µm cell strainer (Corning, NY) to remove debris. To obtain cell pellets and perform cell counts,

BALF suspension was centrifuged (400 x *g*, 4°C for 5 min) and the remaining pellet was re-suspended in 1 mL cold FBS (4°C). After centrifugation, the supernatant was aliquoted into 1.5 mL tubes and stored at -80°C for further analysis (cytokine/chemokine measurement by ELISA and quantification of bacterial amount). Cell number was determined by automatically counting using a Cellometer Bright Field cell counter (Nexcelom Bioscience, Lawrence, MA). For differential BALF cell counts, 0.5 x 10^6 BALF cells were used to make cytospins stained with Diff-Quick (Medion Diagnostics, Medion Diagnostics International Inc., Miami, FL) and a minimum of 400 cells were counted.

Quantifying the number of *Pasteurellaceae* in BALF

BALF were collected and stored as described above. Thereafter, 50 µL BALF was inoculated onto blood agar enriched with 5% sheep blood (bioTRADING) for isolation of *Pasteurellaceae* and incubated overnight at 37°C as described before [3]. The identification of *Pasteurellaceae* was based on morphological characteristics [46, 49] and the number of *Pasteurellaceae* was determined by counting CFUs on each plate and determined in duplicate and represented as CFU/mL BALF.

Identification of *M. haemolytica* in BALF

M. haemolytica-LPS lgG levels in BALF was measured according to manufacturer's instructions (BIO/K-139, Bio-X Diagnostics, Rochefort, Belgium). Negative and positive controls were provided by the same kit. The presence of *M. haemolytica*-LPS lgG was detected at 450nm using a microplate reader (Bio-Rad Laboratories) and showed as the fold of the positive control.

DNA was extracted from BALF using PureLink Genomic DNA Mini Kit (Invitrogen) following the manufacturer’s instructions. Real-time PCR methods for the detection of species-specific genes for *M. haemolytica* were performed using the primers and probes of BactoReal Kit (DVEB02911, Ingenetix GmbH, Vienna, Austria). BactoReal Kit detects the 16S rDNA gene of *M. haemolytica*. A probe-specific amplification-curve at 530nm (FAM channel) indicates the amplification of *M. haemolytica* specific DNA.

Assay mix was prepared in a 20 µL volume that contained 10 µL of DNA Reaction Mix, 3 µL PCR grade water, 5 µL extracted DNA from samples, 1 µL primer, and 1 µL probe. Negative and positive controls were replaced by PCR grade water and positive *M. haemolytica*-DNA in the same kit, respectively. Real-time PCR was conducted on a Real-Time PCR Detection System (Bio-Rad Laboratories).

ELISA measurement

Levels of IL-8 (Mabtech, Nacka Strand, Sweden), IL-6 (Invitrogen, Thermo Fisher Scientific), IL-1β (Invitrogen, Thermo Fisher Scientific) and TNF-α (R&D Systems, Minneapolis, MN) in the supernatants of PBECs or in BALF and blood of calves were determined by using ELISA kits according to manufacturer's instructions. IL-8 (R&D Systems) and IL-6 (BioLegend, San Diego, CA) release in the supernatants of A549 cells after different treatments were also measured by ELISA. The absorbance was measured at 450nm using a microplate reader (Bio-Rad Laboratories).

Clinical scores

Clinical scoring was performed weekly for all calves, according to the Wisconsin calf respiratory scoring system [50], in which a score from 0 to 3 was provided for rectal temperature, coughing, nasal discharge and behavior. The point scale used for respiratory clinical scoring was used as we previously described (Chapter 3, Table 2). Clinical score was calculated as the sum of these 4 scores.

Statistical analysis

Experimental results *in vitro* and *in vivo* are expressed as non-transformed means ± SEM. Statistically significant differences between groups *in vitro* were determined by unpaired Student's t-test, one-way ANOVA, or two-way ANOVA using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA). Results were considered statistically significant when $P < 0.05$ and considered a trend when $P < 0.10$.

In vivo data were analyzed for treatment and time effects with SAS 9.4 (SAS Institute Inc., Cary, NC), using the MIXED procedure, including time as a random statement with calf as unit. For each parameter, the covariance structure was selected based on the lowest AIC and BIC. All analyses included a random effect of pen. For leukocyte counts, the concentration/percentage at arrival (before application of the treatments) was included as a co-variable in the model. Studentized residuals of each model were checked visually on the homogeneity of variance and data were transformed if required to obtain homogeneity of variance. To evaluate differences between treatments, the contrast statement was used, and treatment differences were assessed per timepoint separately. Clinical scores were assessed for treatment and time effects using the GLIMMIX procedure with a multinomial distribution including a random pen effect and potential differences between the treatments were evaluated using the contrast statement per timepoint. Pearson's correlations were applied for the relations within the immune parameters, inflammatory cytokines/chemokines, *Pasteurellaceae* CFUs, and/or *M. haemolytica*-LPS IgG levels in BALF. The Chi-square test was performed for the positivity of *M. haemolytica* in calves.

Differences were considered significant when $P < 0.05$ and considered a trend when $P < 0.10$.

Acknowledgements

This research was performed in the public-private partnership 'CarboKinetics' coordinated by the Carbohydrate Competence Center (CCC, www.cccresearch.nl). CarboKinetics is financed by participating industrial partners Agrifirm Innovation Center B.V., Cooperatie AVEBE U.A., DSM Food Specialties B.V., FrieslandCampina Nederland B.V., Nutrition Sciences N.V., VanDrie Holding N.V. and Sensus B.V., and allowances of The Netherlands Organisation for Scientific Research (NWO). The authors especially thank the VanDrie Group, Mijdrecht, The Netherlands, for providing technical assistance and lung tissue from calves. Research grant funding (No. 201608320245) was received from the China Scholarship Council for Y. Cai.

Supplementary information

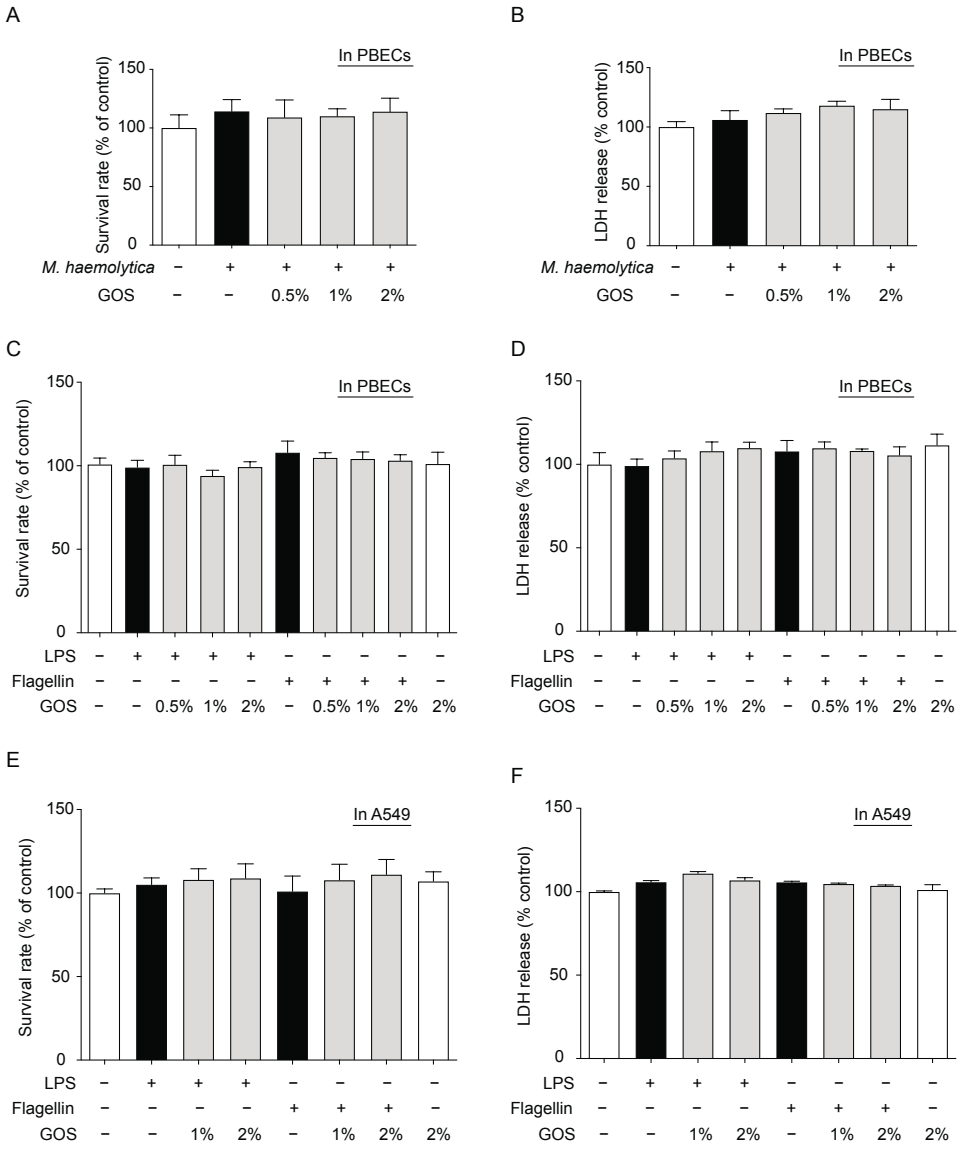


Figure S1. GOS do not affect the survival rates and LDH release in *M. haemolytica*/LPS/flagellin-treated bovine PBECs or in LPS/flagellin-treated human alveolar epithelial (A549) cells. PBECs or A549 cells were incubated with *M. haemolytica* (1 x 10^5 CFU/mL), LPS (10 µg/mL) or flagellin (10 ng/mL) for 24h with or without 24h GOS pretreatment. (**A-D**) Survival rates were determined by the percentage of MTT levels in PBECs and LDH release was assessed in the supernatants of PBECs (n=3 donor calves, one donor calf per experiment). (**E-F**) Survival rates were determined by the percentage of MTT levels in A549 cells and LDH release was assessed in the supernatants of A549 cells (n=3 cell generations, one generation per experiment). Data are presented as means ± SEM. All data shown are representative of three independent experiments.

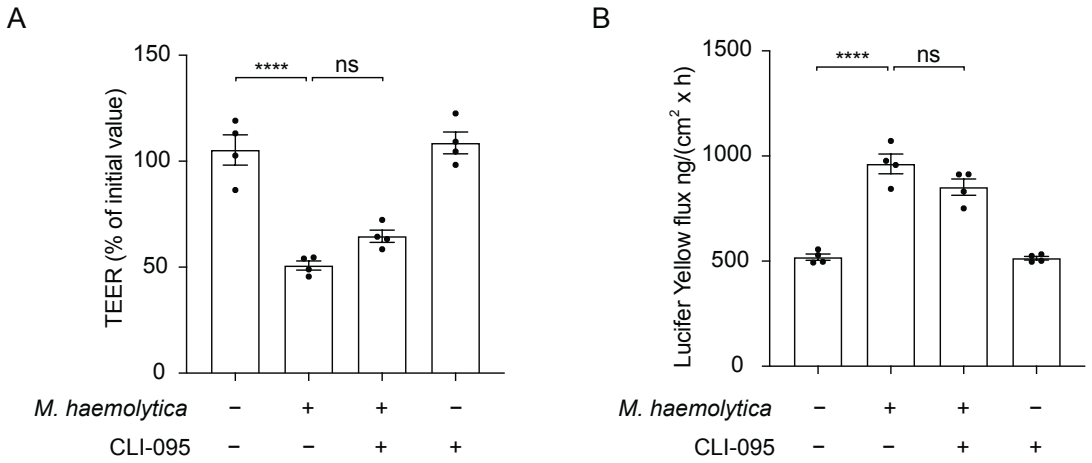


Figure S2. CLI-095 does not affect the *M. haemolytica*-induced disruption of the PBEC monolayer integrity. PBECs were grown on inserts and exposed to *M. haemolytica* (1 x 10^5 CFU/mL) at the apical compartment for 24h with or without 3h pretreatment with CLI-095 (1µM) at the apical and basolateral compartments. After 24h exposure, (**A**) TEER was measured and (**B**) lucifer yellow flux from apical to basolateral compartment was determined. ****P<0.0001; ns=no significance. Data are presented as means ± SEM. All data shown are representative of four independent experiments (n=4 donor calves, one donor calf per experiment).

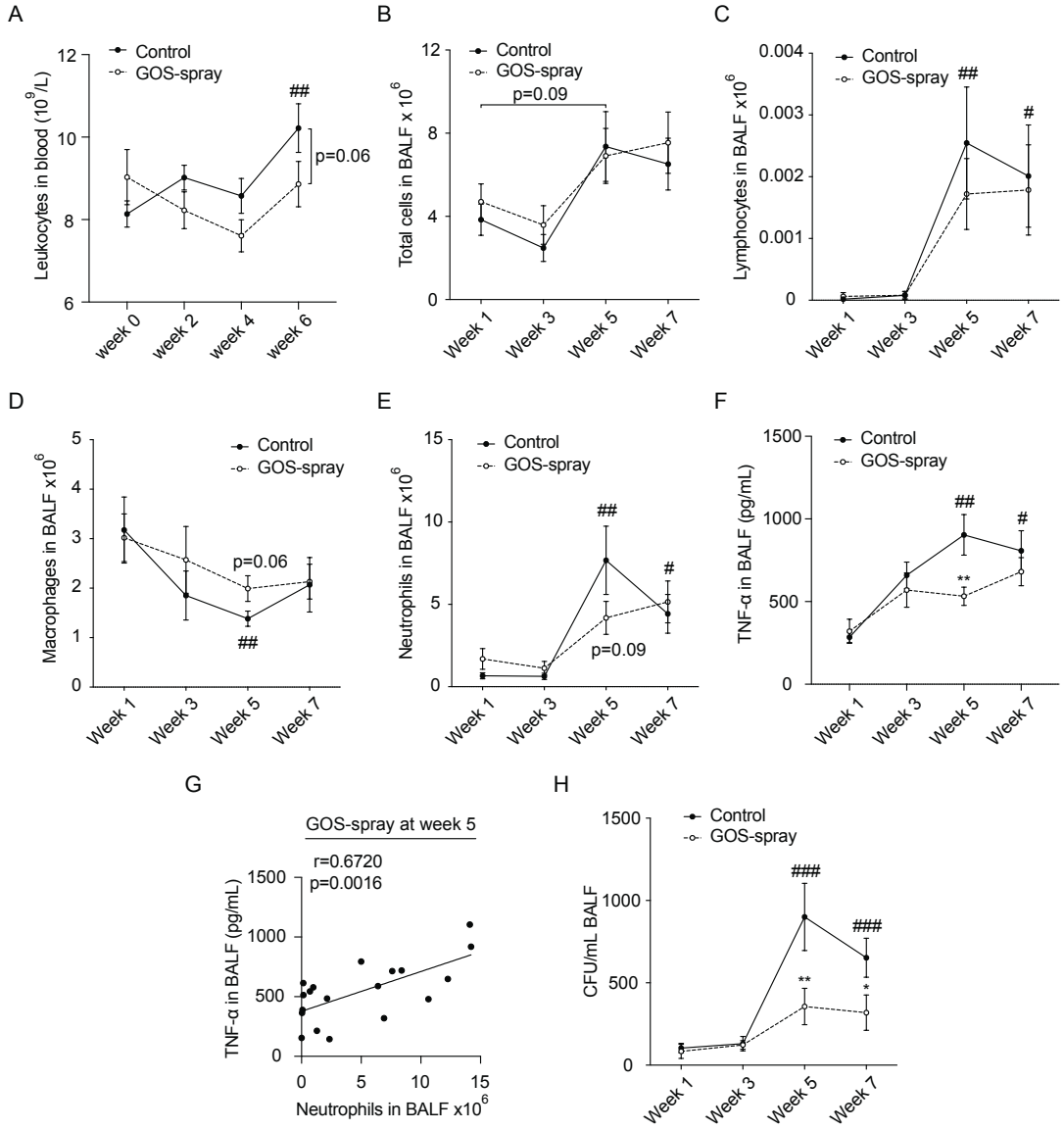


Figure S3. Effect of intranasal GOS on the cell composition, cytokine levels and *Pasteurellaceae* numbers in BALF and/or blood over time. (**A**) Concentrations of leukocytes in the blood were determined for all calves at week 0 (baseline; n=100, 50 calves/group) and for the same subset of calves at week 2, 4 and 6 (n=40, 20 calves/group). (**B-E**) Numbers of total cells, macrophages, neutrophils, and lymphocytes in BALF were determined for the same subset of calves over time (n=40, 20 calves/group). (**F**) TNF-α levels in BALF were measured for the same subset of calves over time by ELISA (n=40, 20 calves/group). (**G**) Correlations between the numbers of neutrophils and the concentrations of TNF-α in BALF of GOS-spray group at week 5. (**H**) BALF was sub-cultured onto the 5% sheep blood agar plates and CFUs of *Pasteurellaceae* were counted for the same subset of calves over time (n=40, 20 calves/group). **P*<0.05; ***P*<0.01 (GOS-spray vs control at that timepoint); #*P*<0.05; ##*P*<0.01; ####*P*<0.0001 (control week 5-7 vs week 0 or 1). Data are presented as means ± SEM.

Table S1. pH values of different concentrations of GOS in PBEC medium.

GOS concentrations	0h incubation	24h incubation
Only medium	8.18	8.20
0.25% GOS	8.20	8.31
0.5% GOS	8.16	8.36
1% GOS	8.29	8.37
2% GOS	8.36	8.45
4% GOS	8.38	8.48
8% GOS	8.35	8.50
16% GOS	8.38	8.52

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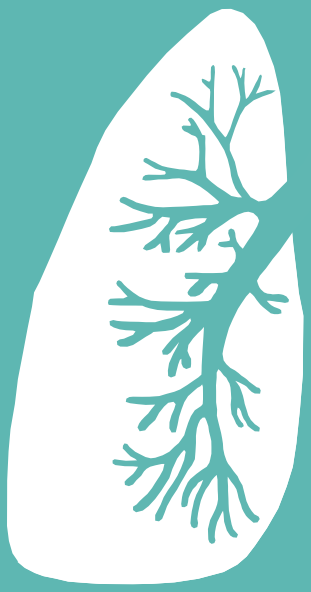

6

Chapter 6

Anti-inflammatory Properties of Fructo-oligosaccharides during Lung Infections in Calves

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(Submitted for publication)

Abstract

Background: Emerging antimicrobial-resistant pathogens highlights the importance of developing novel intervention strategies in respiratory diseases. Fructo-oligosaccharides (FOS) are attractive candidates due to their anti-inflammatory and immunomodulatory potentials.

Objective: To investigate the anti-inflammatory properties of FOS as an oral additive in lung infection models.

Methods: A naturally occurring lung infection model in calves and *M. haemolytica*-treated primary bronchial epithelial cells (PBECs) obtained from calves were used to investigate the anti-inflammatory properties of FOS. Lung scores and cytokine/chemokine levels were investigated in calves. Moreover, the effect of FOS on *M. haemolytica*-treated PBECs were examined by measuring cytokine/chemokine levels, epithelial integrity and tight/adherens junction protein expressions.

Results: FOS reduced the severity of lung lesions and exhibited anti-inflammatory effects both locally and systemically in calves with lung infections. However, FOS did not affect clinical scores. *Ex vivo.* FOS prevented *M. haemolytica*-induced epithelial barrier dysfunction. FOS reduced *M. haemolytica*-induced interleukin (IL)-8, tumor necrosis factor-α, and IL-6 production, as well as p38 mitogen-activated protein kinase and nuclear factor-κB p65 phosphorylation in PBECs. The anti-inflammatory effect of FOS might be due to the interference with Toll-like receptor 5, as observed by the inhibition of flagellin-induced inflammatory responses by FOS.

Conclusions: FOS have anti-inflammatory properties during the natural incidence of respiratory infections but have no effects on clinical symptoms.

Keywords: Respiratory diseases; *Mannheimia haemolytica*; Airway inflammation; Non-digestible oligosaccharides; Primary bronchial epithelial cells

Introduction

Lung infections are a particularly common destructive public health problem in both humans and livestock (calves), associated with high morbidity, mortality, and costs [1-3]. When the defense of the respiratory system is impaired, pathogens quickly invade and colonize the epithelium of the lower respiratory tract, resulting in the development of lung infections [2]. Antibiotics are the main and preferred treatment for lung infections, but the emergence of bacterial resistance necessitates more extensive research on strategies to reduce antibiotic use.

Calves are naturally prone to respiratory infections and have been demonstrated to be a valuable animal model for studying lung infections in general, including high natural prevalence, easy-to-observe respiratory symptoms, and the continuous production of inflammatory mediators in the airways and lungs [2]. Various bacteria and viruses can cause respiratory diseases in calves, among which *Mannheimia haemolytica* is one of the major Gram-negative bacteria that cause lung infections [2, 4]. The airway epithelium forms a complex physicochemical barrier to provide the first line of defense against inhaled pathogens [5]. *M. haemolytica* initiates strategies to impair the airway epithelial barrier to achieve invasion and colonization, including induction of excessive inflammatory responses [6].

Non-digestible oligosaccharides (NDOs) have the potential to prevent respiratory diseases in human and murine species due to their prebiotic, anti-inflammatory and immunomodulatory properties [7-9]. For instance, oral pectin-derived acidic oligosaccharides (pAOS) increased bacterial clearance and reduced tumor necrosis factor (TNF)-α release in mice with *P. aeruginosa*-induced lung infection [8]. The NDO mixture containing fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) prevented (especially respiratory) infections during the first 6 months of age [9] and reduced the frequency of respiratory infections, fever, and antibiotics in the following two years in infants [10]. In addition, some studies suggest that NDOs can regulate the function and signaling of Toll-like receptors (TLRs) [11-13], which orchestrate the inflammatory response to lung infections in both humans and calves by recognizing exogenous pathogen- or endogenous danger-associated molecular patterns [2, 14]. Therefore, NDOs (e.g., FOS) are attractive candidates in the prevention or reduction of lung infections.

Based on these findings, we hypothesized that FOS may alleviate lung infection-induced inflammation in calves. In the current study, we investigated whether FOS could affect the signaling of TLR4 and 5 and the subsequent inflammatory response in primary bronchial epithelial cells (PBECs) as well as

could restore the impaired epithelial barrier function induced by *M. haemolytica*.

Results

Effect of FOS on lung and clinical scores as well as on cell composition in bronchoalveolar lavage fluid (BALF) and blood

All calves were naturally exposed to respiratory pathogens in the environment. Calves were orally administrated twice per day with FOS (0.25%, supplied via the milk replacer) from experimental week 1 till week 8 as an early-life dietary intervention, thereafter the animals were followed from week 9 till 27 without oligosaccharide treatment (**Figure 1A**).

In the present study, there is evidence that lung infections were displayed in these calves from week 5. Increased total cells (increased by 92%; mainly pulmonary leukocytes) in BALF and leukocytes in the blood of control calves were observed at week 5 and 6, respectively (**Figure 1D** and **Figure S1**). Decreased number of macrophages (decreased by 56%) and increased number of neutrophils (increased by 11-fold) and lymphocytes were observed from week 5 in control calves (**Figure 1E-G**). In addition, the clinical scores increased significantly over time with the peak at week 6, indicating a (subclinical) respiratory disease (**Figure 1C**).

FOS-fed calves showed a significant increase in the number of BALF macrophages at week 5, but no effects on other parameters of BALF and blood composition at week 5 were observed (**Figure 1D-G** and **Figure S1**). At week 27, lung scores of all calves were determined after slaughter. Remarkably, dietary FOS showed a lower proportion of moderate/severe lung lesions (18%, $p<0.05$) compared with the control group (44%, **Figure 1B**). However, FOS had no effects on clinical scores, a scoring system for rectal temperature, coughing, nasal discharge and behavior, during the experimental period (**Figure 1C**).

M. haemolytica is one of the main bacteria that contributes to lung infections in calves [15], therefore *M. haemolytica*-LPS IgG levels was investigated in BALF over time. Compared with week 1 (0%), the number of *M. haemolytica* positive calves in control group increased to 80% at week 5, supporting that lung infections exist from week 5. The proportion of *M. haemolytica* positive calves was unaffected by dietary FOS (**Table 1**).

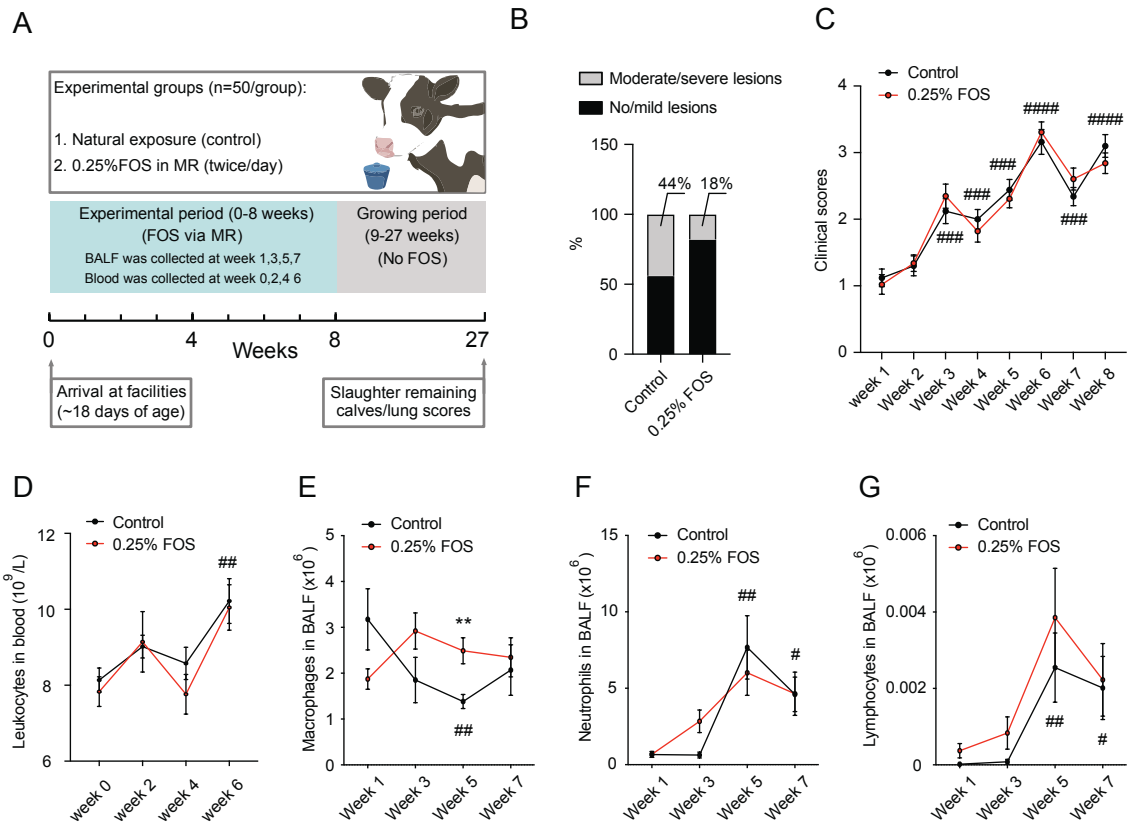

Figure 1. Effect of FOS on lung and clinical scores as well as on cell composition in BALF and blood. (**A**) Timeline and design of the experiment. Calves (n=100, 50 calves/group) were naturally exposed to respiratory pathogens in the environment and treated twice per day with or without FOS orally for 8 weeks during early life followed by 19 weeks (week 9-27) without FOS administration. BALF and blood were collected at experimental week 1, 3, 5, 7 and week 0, 2, 4, 6, respectively. Lung scores were assessed at experimental week 27 at slaughter. (**B**) Proportion of different lung lesions in calves was calculated based on the lung scores (n=75, 36 or 39 calves/group). (**C**) Clinical scores were evaluated over time (n=100, 50 calves/group). (**D**) Leukocyte concentrations in blood were measured at week 0, 2, 4 and 6 (n=40, 20 calves/group). (**E-G**) Number of macrophages, neutrophils, and lymphocytes in BALF was determined at week 1, 3, 5 and 7 (n=40, 20 calves/group). ***P*<0.01 (FOS treatment vs control); #*P*<0.05; ##*P*<0.01 (control week 3-8 vs week 0 or 1). Data are presented as means ± SEM.

Table 1. The number and percentage of calves positive for *M. haemolytica* in BALF over time.

BALF samples (n=20 calves/group)	*M. haemolytica*-LPS IgG detection		*M. haemolytica* positivity (%)		*p-value*	
	Control	FOS	Control	FOS	Time[1]	Treatment[2]
Week 1	0	0	0%	0%	—	—
Week 3	4	4	20%	20%	0.11	>0.99
Week 5	16	14	80%	70%	<0.0001	0.72
Week 7	16	15	80%	75%	<0.0001	>0.99

[1]*P*-values apply to the time effect, week 3, 5, and 7 vs week 1.
[2]*P*-values apply to the treatment effect, FOS vs control.
[3]BALF, bronchoalveolar lavage fluid; FOS, fructo-oligosaccharides; LPS, lipopolysaccharides.

Effect of FOS on cytokine/chemokine levels in BALF and blood

The inflammatory response in the lungs was investigated by measuring cytokines/chemokines in BALF at week 5. FOS tended (p=0.07) to inhibit the concentrations of TNF-α in BALF at week 5 (**Figure 2B**). In addition, FOS significantly suppressed the concentrations of IL-8, IL-6, and IL-1β in BALF at week 5 (**Figure 2A**, **C** and **D**). The same cytokines/chemokines were measured in blood to investigate the effect of FOS on systemic inflammation at week 4 and 6. In control calves, the IL-8 and TNF-α levels in blood were significantly increased at week 6 compared to week 4, while IL-6 and IL-1β concentrations were slightly reduced (**Figure 2E-H**). Oral FOS reduced the concentrations of IL-8, IL-6 and IL-1β in blood at both week 4 and 6, while no effects on TNF-α levels were observed (**Figure 2E-H**).

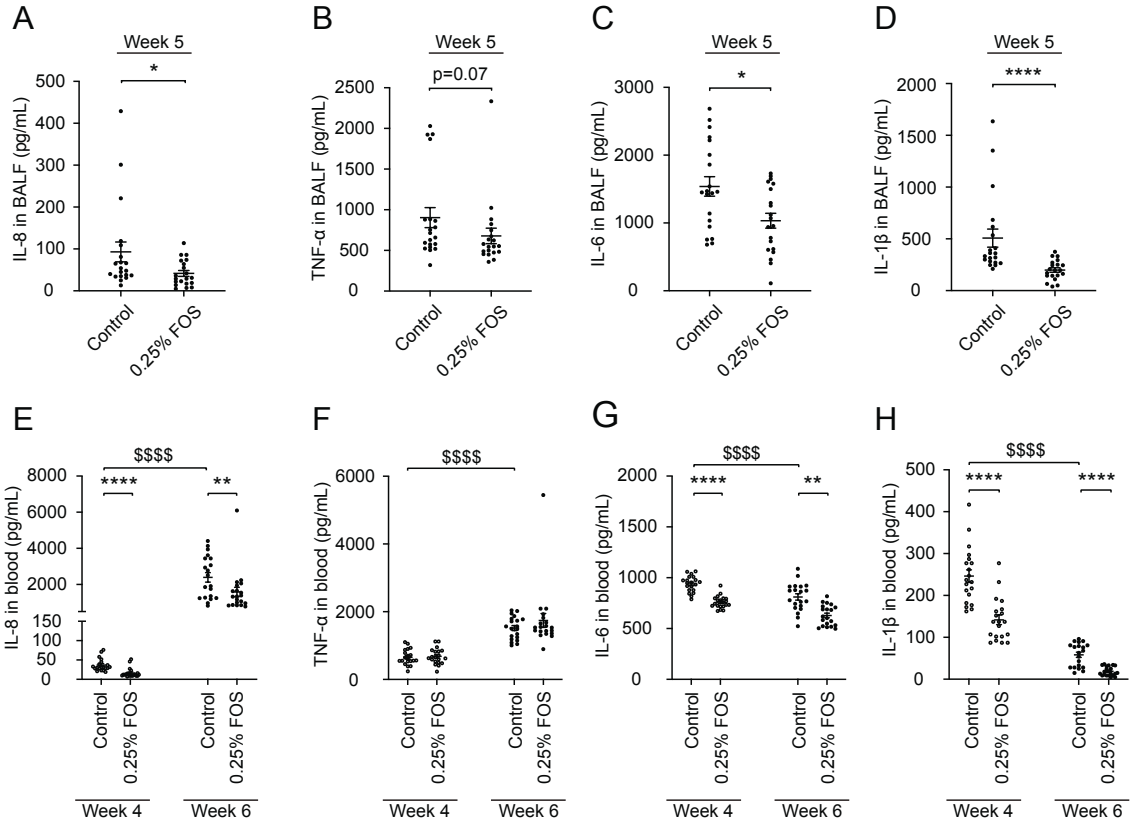

Figure 2. Effect of FOS on cytokine/chemokine levels in BALF and blood. (**A**-**D**) IL-8, TNF-α, IL-6, and IL-1β concentrations in BALF were measured at week 5 (n=40, 20 calves/group). (**E**-**H**) IL-8, TNF-α, IL-6, and IL-1β concentrations in blood were measured at week 4 and 6 (n=40, 20 calves/group). **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 (FOS treatment vs control); $$$$*P*<0.0001 (control week 6 vs week 4). Data are presented as means ± SEM. Each dot represents one calf.

FOS increase the growth of *M. haemolytica in vitro*

Recently, our group and others found that NDOs (e.g., GOS and FOS) have the ability to directly affect the growth of pathogens [13, 16]. Here, the effects of FOS were tested on the growth of *M. haemolytica,* an important pathogen might be involved in lung infections in the present *in vivo* study. Bacterial growth assays (minimal inhibitory/bactericidal concentration assays) showed that 1% and 2% FOS significantly increased the growth of *M. haemolytica* after 24h incubation, while FOS <1% did not (**Figure 3**).

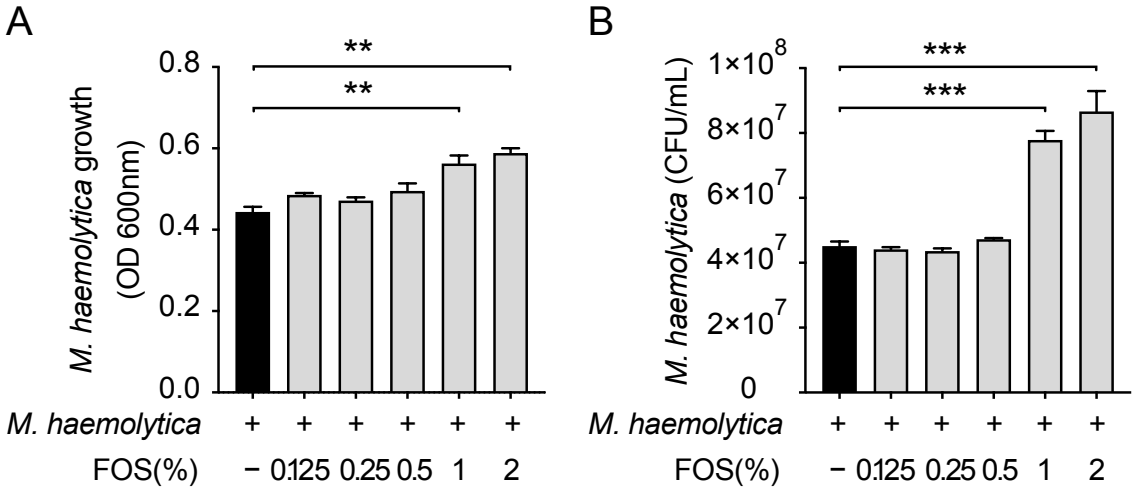

Figure 3. FOS increase the growth of *M. haemolytica in vitro.* *M. haemolytica* was incubated with FOS for 24h, thereafter supernatants were diluted and inoculated onto 5% sheep blood agar plates overnight. (**A**) *M. haemolytica* growth (OD600nm) was measured after 24h FOS treatment. (**B**) *M. haemolytica* number on the sheep blood agar plates after subculture were determined by counting CFUs. ***P*<0.01; ****P*<0.001 (FOS treatments vs *M. haemolytica* group). Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5).

FOS suppress *M. haemolytica*-induced inflammation in PBECs

Lung infection and inflammation caused by *M. haemolytica* are dependent on the adhesion to and invasion of epithelial cells, which is the first line of defense against respiratory pathogens [2, 17]. To better understand and intervene in lung infections, we harvested and developed an *ex vivo* infection model based on PBECs of healthy bovine lungs [6]. FOS concentrations of 0.5% and lower were used in the following experiment since these concentrations did not affect *M. haemolytica* growth as shown before (**Figure 3**).

M. haemolytica induced the release of IL-8, TNF-α and IL-6 in PBECs, while the release was significantly inhibited by pretreatment with 0.5% FOS (**Figure 4A-C**). Moreover, *M. haemolytica* with or without the pre-administration of FOS did not affect lactate dehydrogenase (LDH) release in PBECs (**Figure 4D**).

In addition to adhesion to and entry into epithelial cells, *M. haemolytica* also release virulence factors to accelerate epithelial inflammation [2, 17]. Here, the supernatants (*M. haemolytica*-cultured supernatant, MHS) were collected from *M. haemolytica* alone incubation for 24h. These MHS were filtered to remove *M. haemolytica* and used as a stimulant for PBECs to investigate whether the

observed anti-inflammatory effect of FOS (**Figure 4**) is related to direct interaction with *M. haemolytica* or whether virulence factors and other mediators also play a role in this process. MHS successfully induced the release of IL-8, TNF-α and IL-6 in PBECs after 24h incubation (**Figure S2A-C**), while did not affect the LDH release (**Figure S2D**). Interestingly, pre-incubation with FOS (24h) also caused a significant inhibition in MHS-induced IL-8, TNF-α and IL-6 levels in PBECs, while no effects in the FOS alone group were observed (**Figure S2**).

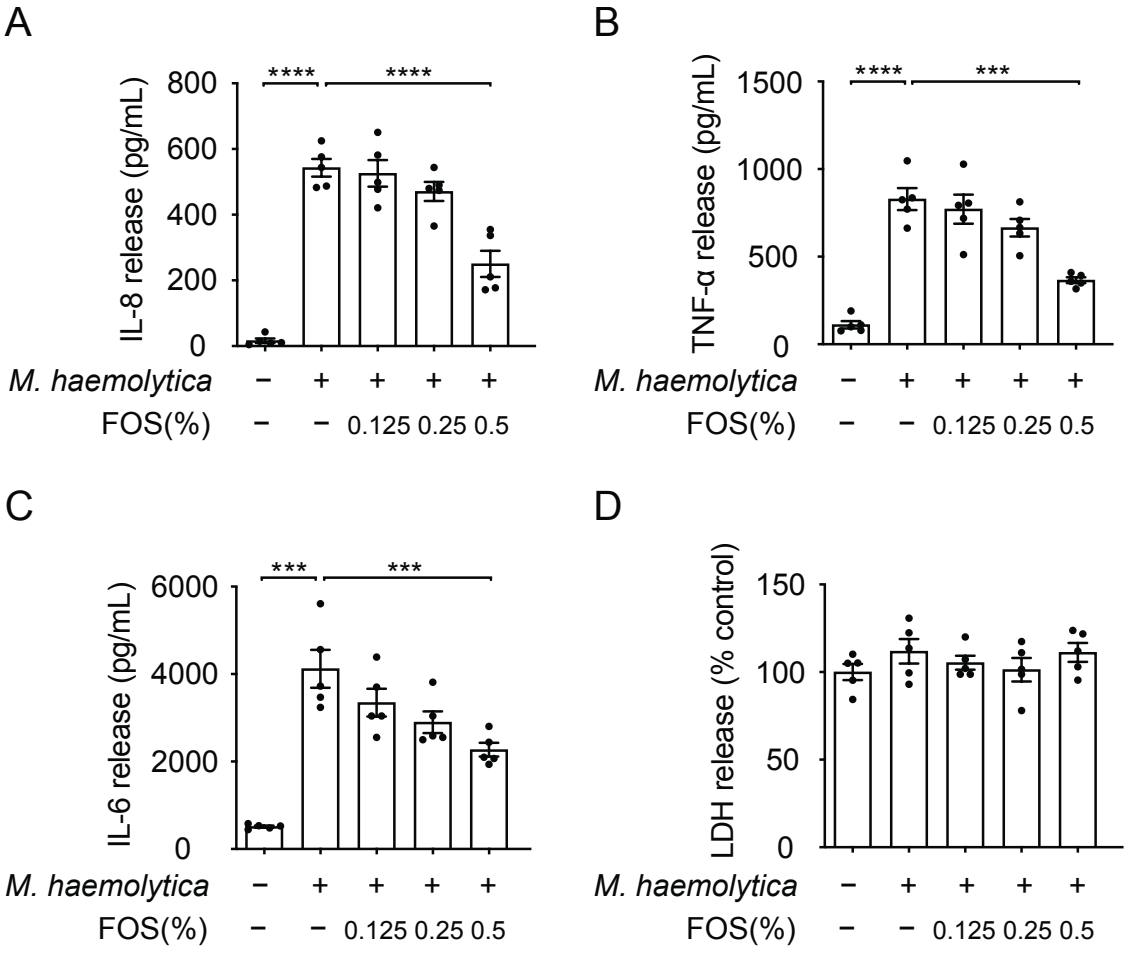

Figure 4. FOS suppress *M. haemolytica*-induced inflammation in PBECs. PBECs were incubated with *M. haemolytica* (1 x 10^5 CFU/mL) for 24h with or without 24h pretreatment with FOS. (**A**-**C**) IL-8, TNF-α and IL-6 levels in the supernatants of PBECs were measured by ELISA. (**D**) LDH release was assessed in the supernatants of PBECs. ***P<0.001; ****P<0.0001. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves).

FOS inhibit *M. haemolytica*-induced barrier dysfunction in PBECs

Another characteristic of *M. haemolytica*-treated airway epithelial cells is the disruption of epithelial barrier function [6, 17]. In the present study, *M. haemolytica* exposure (24h) resulted in a significant decrease in transepithelial electrical resistance (TEER) (**Figure 5A**), which facilitated the translocation of lucifer yellow from the apical to the basolateral compartment (**Figure 5B**) and a reduction in the protein level and distribution of the tight junction protein ZO-1 and the adherens junction protein E-cadherin (**Figure 5C** and **D**).

The 24h pretreatment with 0.5% FOS prevented the *M. haemolytica*-induced TEER decrease (**Figure 5A**) and increase in lucifer yellow flux (**Figure 5B**) as well as the reduction of ZO-1 and E-cadherin expression (**Figure 5C-E**).

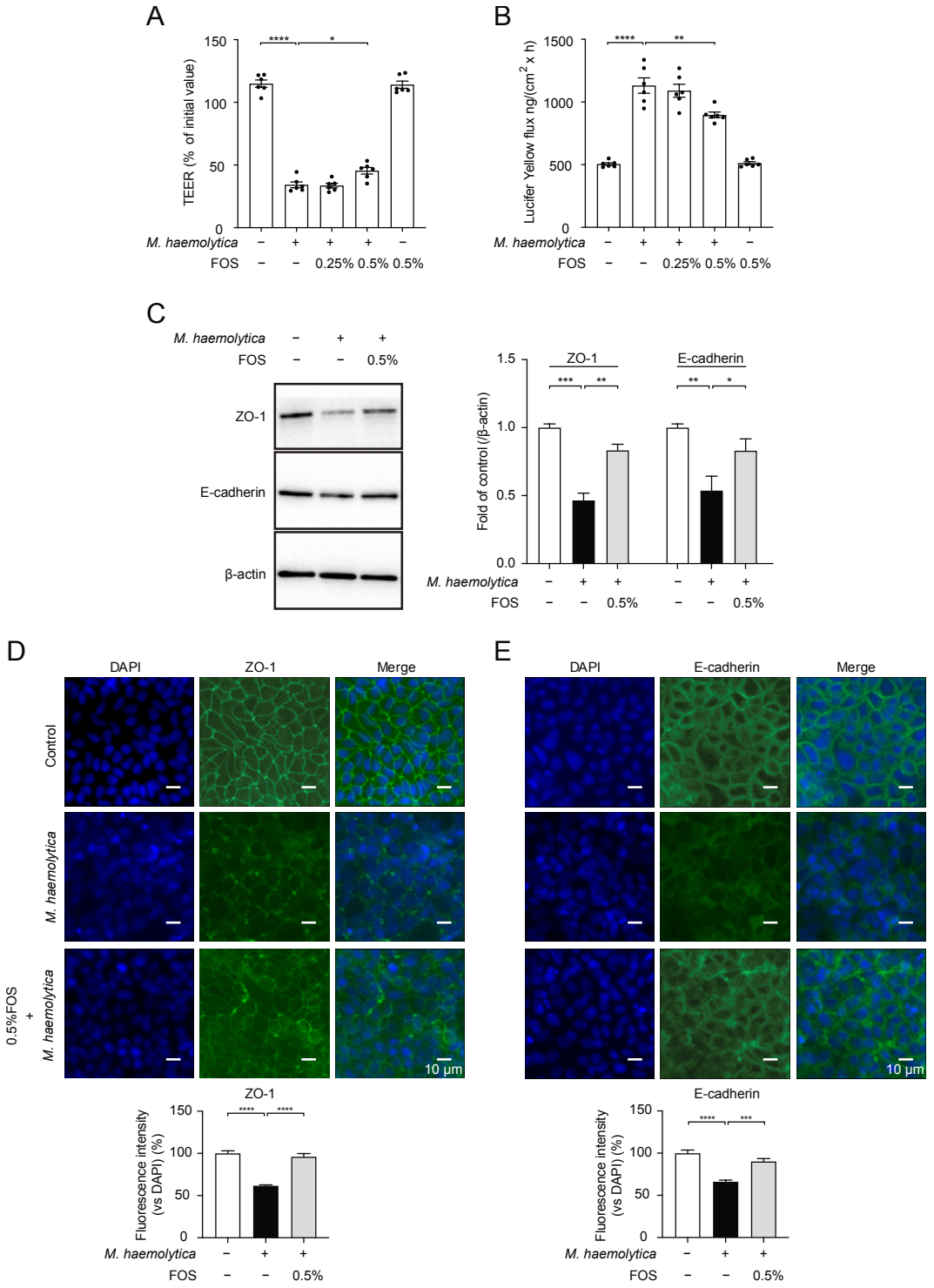

Figure 5. FOS inhibit *M. haemolytica*-induced barrier dysfunction in PBECs. PBECs were grown on inserts and exposed to *M. haemolytica* (1 x 10^5 CFU/mL) at the apical compartment with or without 24h pretreatment with FOS at the apical and basolateral compartments. (**A**-**B**) After 24h exposure, the TEER was measured and lucifer yellow flux from apical to basolateral compartment was determined. The data of control and *M. haemolytica* groups in Figure 5A and B were reused in the control and *M. haemolytica*

groups in Figure 4A and B of Chapter 5. (**C**) The immunoblots were obtained with ZO-1, E-cadherin, and β-actin (protein loading control). (**D**-**E**) Cellular expression of ZO-1 and E-cadherin in PBECs was assessed by immunofluorescent staining and quantified as a percentage of fluorescence intensity. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5-6 donor calves).

FOS suppress *M. haemolytica*-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB p65 in PBECs

Activation of MAPK and NF-κB signaling pathways played an important role in the *M. haemolytica*-induced inflammation and barrier dysfunction in PBECs as observed by the phosphorylation of p38, ERK1/2, JNK1/2 MAPK and NF-κB p65 in our previous study [6]. Here, the pretreatment with 0.5% FOS showed a decrease in the phosphorylation of p38 MAPK and NF-κB p65 in PBECs after *M. haemolytica* exposure (**Figure 6**). However, FOS did not affect the phosphorylation of ERK1/2 and JNK1/2 MAPK in *M. haemolytica*-treated PBECs (**Figure 6**).

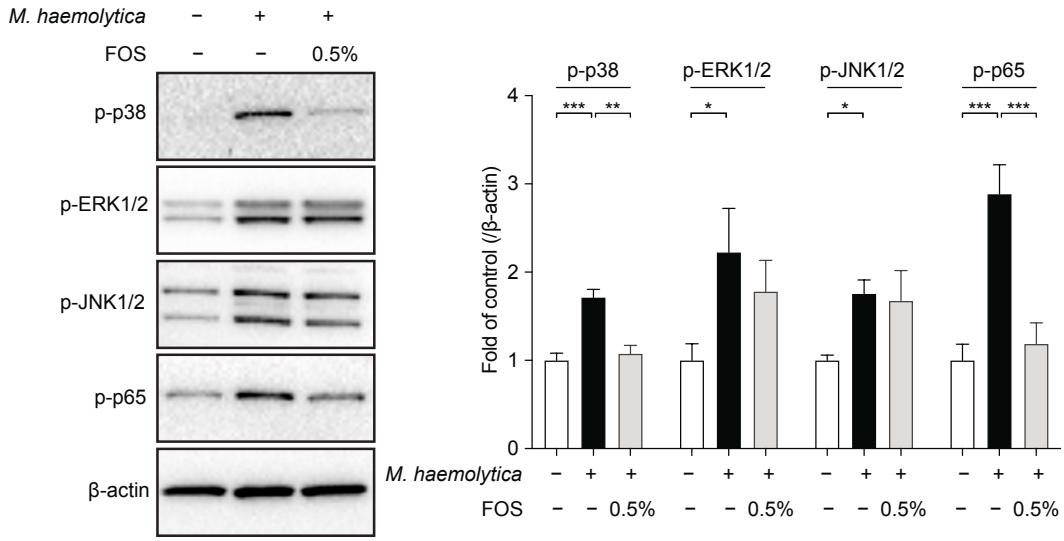

Figure 6. FOS suppress *M. haemolytica*-induced phosphorylation of p38 MAPK and NF-κB p65 in PBECs. PBECs were grown on inserts and exposed to *M. haemolytica* (1 x 10^5 CFU/mL) at the apical compartment with or without 24h pretreatment with 0.5% FOS at the apical and basolateral compartments. Phosphorylation of p38, ERK1/2, JNK1/2 MAPK and NF-κB p65 were determined by immunoblot and results were shown as a fold of control. **P*<0.05; ***P*<0.01; ****P*<0.001. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves).

Anti-inflammatory effect of FOS might be related to the interference with TLR5

Recent studies suggested that NDOs can exert anti-inflammatory effects via TLRs (e.g., TLR4 and TLR5) in epithelial cells [13, 18]. Therefore, we assume that FOS may affect the cascade signaling of TLR4 or 5, the important TLRs associated with bacterial lung infections [14, 19], preventing the inflammatory responses. In this study, commercial LPS (TLR4 ligand) derived from *E. coli* and flagellin (TLR5 ligand) derived from *P. aeruginosa* were used to verify the anti-inflammatory effect of FOS related to these specific TLR targets in PBECs.

Interestingly, FOS suppressed flagellin- but not LPS-induced release of IL-8, TNF-α and IL-6 (**Figure 7A-C**) in PBECs, while it did not affect the LDH release (**Figure 7D**).

In addition, this effect of FOS in PBECs was also confirmed in a human alveolar basal epithelial cell (A549), where FOS significantly inhibited the flagellin-induced release of IL-8 and TNF-α, but not IL-6 in A549 cells (**Figure S3D-F**). FOS did not affect the LPS-induced cytokine/chemokine release and corresponding LDH release in A549 cells (**Figure S3A**, **B**, **C**, and **G**).

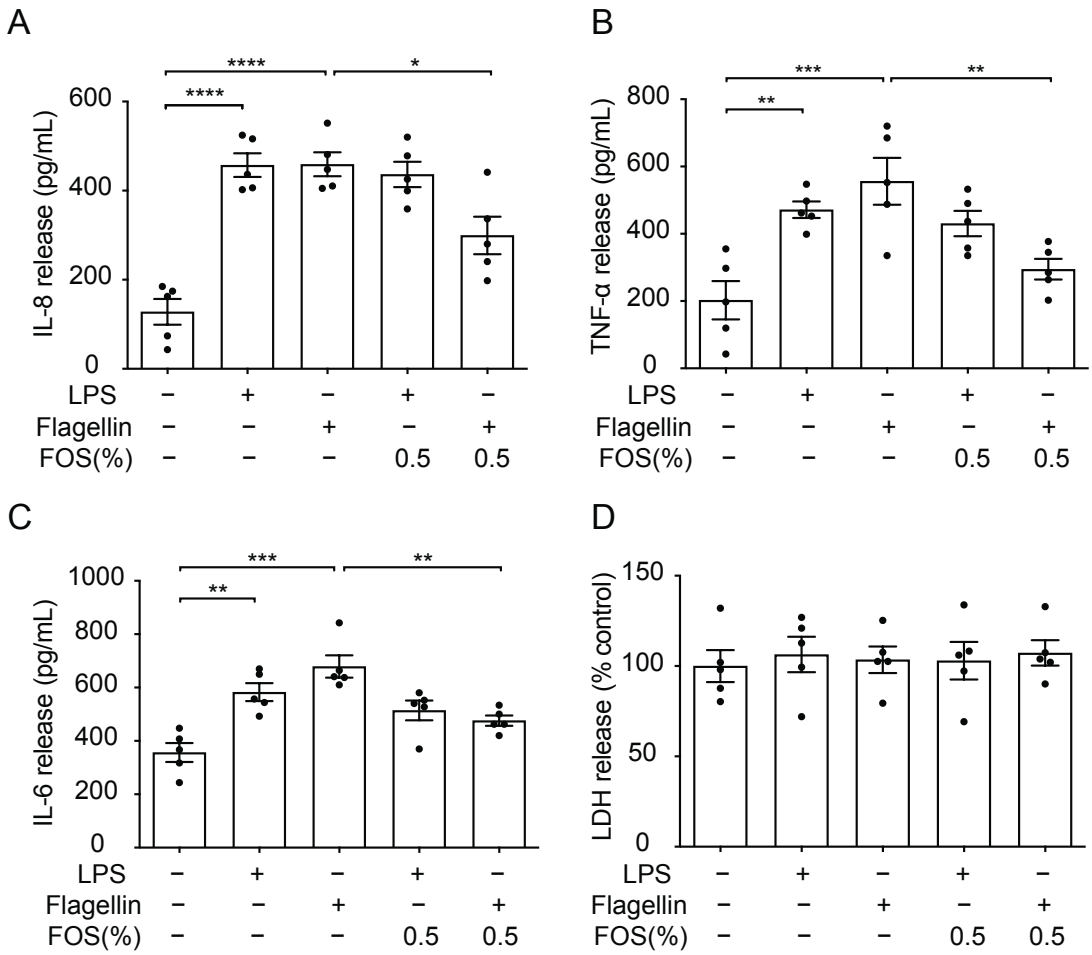

Figure 7. Anti-inflammatory effects of FOS in LPS- or flagellin-treated PBECs. PBECs were incubated with LPS (10 µg/mL) or flagellin (10 ng/mL) for 24h with or without 24h pretreatment with FOS. (**A**-**C**) IL-8, TNF-α and IL-6 levels in the supernatants of LPS- or flagellin-treated PBECs were determined by ELISA. (**D**) LDH release was assessed in the supernatants of PBECs. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves).

Discussion

This study investigated whether FOS could reduce lung infection and subsequent inflammation. Here, calves naturally exposed to environmental pathogens and *M. haemolytica*-stimulated PBECs cloud be used to investigate the anti-inflammatory properties of FOS. Different studies demonstrated that NDOs (e.g., FOS, GOS) have beneficial effects on other organs than the intestine, including

the airways [7, 20], and are therefore a suitable candidate to investigate as early dietary intervention to reduce lung infections.

The calf is considered to be a suitable animal model for studying lung infections: the prevalence of pneumonia is extremely high, involving airborne and close contact transmission [2]. However, during natural exposure (in the current study) or in inoculated lung infection models, clinically healthy or asymptomatic individuals are present [21, 22]. This could be the reason why the effect of oral FOS on BALF cell composition and clinical scores might be underestimated. In addition, the application of antibiotic strategy against lung infections for all groups may (partly) reduce the severity of lung infections which could interfere with obtained results. Additionally, the insensitivity of clinical scores to diagnose (subclinical) lung infections may also lead to contrasting results as compared to the measurements of cell composition and cytokine/chemokine levels in BALF/blood [23].

Although calves stopped receiving the dietary intervention after experimental week 8, the FOS-treated group showed reduced severity of lung lesions after 27 weeks, which might be due to the FOS-induced changes in the gut microbiota composition in calves and the interplay between the microbiota and the immune system [24]. The gut microbiota changes caused by weaning and/or other stressors is an important reason for the development of respiratory diseases in calves and children [4, 25, 26]. FOS have the ability to selectively stimulate the growth of commensal bacteria in the intestine, such as *Bifidobacteria* and *Lactobacillus*, which play an important role in preventing infections [13]. In a randomized trial with children, consuming long-chain FOS (lcFOS) resulted in a higher relative abundance of *Bifidobacteria* and *Lactobacillus* in feces and a fewer incidence of febrile episodes and sinusitis [27]. In addition, the promising anti-inflammatory effects of FOS both systemically and locally as observed by a decrease in IL-8, IL-6, and IL-1β levels in BALF and blood might be also related the crosstalk between the gut microbiota and the lungs, possibly work through the gut-lung axis [24]. Future studies based on the analysis of the gut and lung microbiota in infected calves after NDO intervention might be interesting to further understand the microbiota-driven pathophysiology and inflammation via the gut-lung axis.

Most reported studies focus on the well-known intestinal protection and prebiotic effects of oligosaccharides, while there are few studies related to respiratory diseases. For instance, dietary intervention with 1% GOS suppressed the increased BALF leukocyte numbers and CCL5 and IL-13 levels in a murine house dust mite-induced asthma model [28]. Additionally, a mixture of

GOS/lcFOS/pectin supplementation decreased the BALF neutrophil numbers in mice with LPS-induced lung emphysema [7]. Moreover, dietary pAOS recruited polynuclear leukocytes and macrophages, and decreased TNF-α release in the lung, leading to increased bacterial clearance after *P. aeruginosa* infection in mice [8]. In the present study, dietary FOS increased total cells (mainly macrophages) and macrophages in BALF at week 3 and 5, respectively. In particular, the increased macrophages might be helpful in contributing to bacterial clearance after lung infections in calves. It has been reported that alveolar macrophages, as resident immune cells in the epithelial mucosa of the lower respiratory tract, play an important role in respiratory defense, including rapid response to phagocytose pathogens and cell debris [2].

Increasing evidence shows that different NDOs have the capacity to be absorbed into the systemic circulation after oral administration [29, 30]. In an experiment in rats, different HMOs (2'-fucosyllactose, 2′-FL; 6'-sialyllactose, 6′-SL; and lacto-N-neotetraose, LNnT) administered orally were detected in blood and urine [29]. Our previous study with piglets reported that after oral ingestion of GOS (0.8% GOS, daily), around 0.1% GOS (16-23 μg/mL) was observed in blood serum [31]. Moreover, Eiwegger *et al*. showed *in vitro* evidence for transport of prebiotic oligosaccharides, like FOS, across the intestinal epithelial layer [32]. These studies suggest that FOS might reach the lungs (bronchus) through blood circulation, resulting in direct inhibition of inflammation and protection of epithelial barrier function in the airways.

Previously studies often focus on the beneficial effects of FOS on intestinal cells, while here we investigated the direct effects of FOS on airway epithelial cells. 0.5% FOS inhibited the release of cytokines/chemokines (IL-8, IL-6, and TNF-α) in *M. haemolytica*-stimulated PBECs, indeed suggesting a possible direct anti-inflammatory capacity of FOS in the airways. Concentrations higher than 0.5% FOS were not investigated in this *in vitro* study as >0.5% FOS promoted the growth of *M. haemolytica*. It can be suggested that FOS might be used as beneficial carbon source for *M. haemolytica* growth and survival *in vitro*. Additionally, we can exclude that the anti-inflammatory effects of FOS observed *in vitro* are related to *M. haemolytica* growth inhibition or bacterial killing. The accumulation of inflammatory cytokines (e.g., IL-6 and TNF-α) caused by infections in the airways is one of the important causes for the sustained impairment of epithelial barrier function [2, 33-35]. We showed that FOS have the ability to inhibit the levels of these cytokines *in vitro* and *in vivo*, thereby protecting the integrity of the epithelial barrier, as observed by increased TEER values and expression of tight/adherens junction proteins, and decreased lucifer yellow flux in PBECs. In addition, FOS may have direct protective effects on epithelial barrier

by modulation of host cell signaling in the epithelium [36]. Our previous study indicated that 2% FOS could significantly modulate deoxynivalenol-induced epithelial barrier disruption in Caco-2 cells [37].

In studies describing the anti-inflammatory properties of NDOs, the potential role of TLR modulation, such as TLR4 and 5 regulations, in cytokine production has been demonstrated [11, 18]. It has also been reported that specific NDOs, such as FOS and GOS, may affect the inflammatory signal transduction pathways mediated by TLRs/NF-κB and secondarily by MAPK [12]. In the present study, FOS were able to inhibit *M. haemolytica*-induced release of cytokines/chemokines (IL-8, IL-6, and TNF-α) and phosphorylation of NF-κB p65 and p38 MAPK in PBECs. Our studies with MHS-stimulated PBECs showed that the anti-inflammatory effects of FOS are not related to direct interaction with *M. haemolytica,* but other factors, such as virulence factors and other mediators may play a role in this process. We observed that FOS inhibited flagellin- but not LPS-induced inflammatory responses, which might be related to the interference with TLR5 signaling in PBECs and A549 cells. Specific HMOs (2′-FL, 6′-SL, LNnT, and 3-fucosyllactose) also suppressed flagellin-induced TLR5 activation in HEK-Blue hTLR5 cells, but did not affect LPS-induced TLR4 activation in HEK-Blue hTLR4 cells [18]. There is no evidence in the literature that *M. haemolytica* produce flagellin, therefore FOS might inhibit the inflammatory response caused by other virulence factors (e.g., LPS) by interfering with TLR5 signaling. It could be possible that higher FOS concentrations are needed to modulate the LPS-induced inflammatory response in PBECs. A recent study in TLR5-deficient mice showed that a decreased cytokine expression in the lungs was observed after LPS exposure in the lungs, which might be due to the participation of TLR5 in TLR4-dependent signaling [19].

Overall, to our knowledge, this is the first study that use FOS to relieve lung infections in calves. Interestingly, FOS have the capability to reduce lung inflammation in infected calves systemically and locally and prevent *M. haemolytica*-induced epithelial barrier dysfunction and inflammatory responses *ex vivo,* which might be due to the interference with TLR5 signaling. The suppression of infection-induced inflammation by FOS, may contribute to reducing the future use of antibiotics in animals and humans.

Methods

Animal experiment design

This experiment was conducted under the Dutch Law on Animal Experiments in accordance with EU Directive 2010/63 at the research facilities of the VanDrie Group (Scherpenzeel, The Netherlands) and was approved by the Animal Care and Use Committee of Wageningen University (AVD1040020185828, Wageningen, The Netherlands).

The experiment consisted of 2 periods, an experimental period, and a growing period. In period 1 (experimental week 1 to 8), 100 male Holstein Friesian calves (~18 days of age; 43.2 ± 0.33 kg, means ± SEM) of German origin were used and assigned randomly to 2 groups supplied twice a day with calf milk replacer (MR) without (control) or with 0.25% FOS (92% purity based on dry matter, DP 2-8, Frutalose OFP, Sensus, The Netherlands). Most of the measurements were performed in period 1 for all calves or for a subset of calves. The subset of calves included 2 calves per pen and 20 calves per group and was selected on body weight at arrival, closest to the average body weight of all calves at arrival. In period 2 from week 9 to 27, calves stop receiving oligosaccharide treatments and received the same diet as control. At the end of period 2, all calves were slaughtered, and lungs were scored.

During these periods, all calves were naturally exposed to pathogens in the environment. Individual antibiotic treatment was applied when required based on clinical signs of illness as assessed by the animal caretakers. The number of applied individual antibiotic treatments did not differ between the control and FOS groups ($P > 0.1$). Group antibiotic treatment was applied equally to all groups if 10% of the calves had been treated within 5 days, or if 5% of the calves had become ill within 24h, or when the situation required group antibiotics in the expert judgement of a veterinarian.

The *in vivo* study described in this article was part of a large calf trial, including 300 calves randomly assigned to a control group, 0.25% FOS group, and 4 other groups with different (dietary) interventions (50 calves/group). In accordance with the purpose of this study, investigating the effect of oral FOS supplementation on lung infection, we reported here the results of the analyses of the control and 0.25% FOS groups (100 of 300 calves) of this large calf trial.

Experimental diets

The MR mainly contained 527 g/kg whey powder, 35 g/kg lactose, 52 g/kg delactosed whey powder, 50 g/kg whey protein concentrate, 60 g/kg soy protein

concentrate, 50 g/kg soluble wheat protein, 3 g/kg pea fiber, 179.4 g/kg fat sources, 9.7 g/kg calcium formate, 2 g/kg citric acid, 4 g/kg sodium bicarbonate, 3.5 g/kg mono ammonium phosphate, 9.8 g/kg lysine, 2.4 g/kg methionine, 1.3 g/kg threonine, 0.2g/kg aroma and 10 g/kg premix. FOS administered via the MR were included at the expense of lactose, corrected for the purity and DM of the FOS product used.

Lung scores

Calf lungs were scored at slaughter and obtained by using a scoring system adapted from Leruste *et al.* [38]. Briefly, the observer (veterinarian) visually examined each lung (cranial and ventral lobes) evaluating signs of pneumonia. Each examined lung was classified according to a 4-point scale for pneumonia from healthy lung (score 0) to severe lesions (score 3). Score 0 for healthy lungs (pale orange color with no sign of pneumonia), score 1 for minimal or mild lesions (one spot of grey-red discoloration), score 2 for moderate lesions (one larger or several small spots of grey-red discoloration with a total surface of less than 1 lobe), and score 3 for severe lesions (grey-red discoloration area of at least one full lobe and/or presence of abscesses). The results are shown as a percentage of the total calves with different severity of pneumonia.

Measurement of *M. haemolytica* positivity in BALF

BALF were collected and stored as described above. To investigate the positivity of *M. haemolytica*, *M. haemolytica*-LPS IgG levels were measured in BALF according to manufacturer's instructions (BIO/K-139, Bio-X Diagnostics, Rochefort, Belgium).

Bacterial growth conditions

M. haemolytica (isolated from a pneumonic bovine lung) was kindly provided by Prof. Jos van Putten (Utrecht University, Utrecht, The Netherlands). *M. haemolytica* was incubated overnight at 37°C in 5% sheep blood agar (bioTRADING, Mijdrecht, The Netherlands).

Quantification of *M. haemolytica* growth

For bacterial quantification, *M. haemolytica* (1×10^5 CFU/mL) was cultured in 96-well plates with or without FOS (0.125%, 0.25%, 0.5%, 1%, or 2%) for 24h. After incubation, *M. haemolytica* growth were determined by measuring the turbidity of supernatants at OD600nm using a microplate reader (Promega Corp., Madison, WI).

Then, the supernatants were diluted 50,000-fold and sub-cultured onto 5% sheep blood agar plates overnight at 37°C. Bacterial numbers were determined

by counting CFUs of each plate.

Preparation of *M. haemolytica*-cultured supernatant

M. haemolytica (1 x 10^5 CFU/mL) were cultured in RPMI-1640 medium containing 10% FBS (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 24h on 6-well plates (Corning). Thereafter, the supernatants were collected and filtered with a 0.25 µm filter (GE Healthcare, Chicago, Illinois).

Isolation and culture of PBECs

Isolation and culture of PBECs were conducted as previously described [6]. Briefly, PBECs were isolated from bovine bronchial epithelium obtained from the lungs of freshly slaughtered calves aged 6-8 months, provided by Ekro bv (Apeldoorn, The Netherlands). After digesting of the bronchial epithelium, PBECs were collected and grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and attached to collagen-coated plates in serum-free RPMI-1640 medium for 2-3 days until reaching near-confluence (70-90%) and then replaced with RPMI-1640 medium containing 10% FBS, 1% L-glutamine, 1% MEM NEAA, and 1% penicillin–streptomycin (Sigma-Aldrich, Zwijndrecht, The Netherlands) for future culture and experiments as described before [6].

Human alveolar epithelial cell line culture

Human Type II alveolar epithelial cells (A549; ATCC, Manassas, VA) were grown in Ham's F-12K Medium (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS and 1% penicillin–streptomycin (Sigma-Aldrich) in 5% CO_2 at 37°C.

PBEC and A549 treatments

PBECs were cultured at a density of 1 x 10^6 cells/mL in 96-well plates (Corning, NY, US) pre-coated with collagen, fibronectin and BSA as we previously described [6]. After reaching near-confluence, these PBECs were first pretreated with different concentrations of FOS (0.125%, 0.25%, or 0.5%) for 24h and then were stimulated with LPS (10 µg/mL; isolated from E. *coli* O111:B4, Sigma-Aldrich), flagellin (10 ng/mL; isolated from *P. aeruginosa*, InvivoGen, San Diego, CA), *M. haemolytica* (1 x 10^5 CFU/mL) or MHS for 24h. After stimulation, supernatants were collected and stored at -20 °C until future analysis.

A549 cells were cultured at a density of 0.5 x 10^5 cells/mL in 96-well plates. After reaching near-confluence, A549 cells were pretreated with 0.5% FOS for 24h prior to 24h LPS (10 µg/mL; isolated from *E. coli* O111:B4, Sigma-Aldrich) or flagellin (10 ng/mL; isolated from *P. aeruginosa*, InvivoGen) stimulation. After stimulation, supernatants were collected and stored at -20 °C until analysis.

TEER measurement and paracellular tracer flux assay

PBECs (1 x 10^6 cells/mL, 300 µL) were added to the apical compartment of the permeable 0.3 cm^2 high pore density polyethylene membrane transwell inserts (Corning) placed in a 24-well plate and 700 µL cell culture medium (RPMI-1640 containing 10% FBS) was added to the basolateral compartment. After that, PBECs were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. TEER of PBECs was measured by a Millicell-ERS Volt-Ohm meter (Millipore, Merck, Darmstadt, Germany) every 2 days. The culture medium from the basolateral and apical compartment was refreshed after TEER measurement and experiments started at day 11 when sustained TEER values around 600 Ω·cm^2 were reached as we described before [6].

PBECs were incubated with *M. haemolytica* (1 x 10^5 CFU/mL) from the apical side with or without 24h pretreatment with FOS at both the apical and basolateral compartments. TEER was measured at 0 and 24h after *M. haemolytica* exposure. Thereafter, a membrane-impermeable molecule, lucifer yellow (molecular mass of 0.457 kDa, 20 µg/mL; Sigma-Aldrich), was added to the apical compartment for 4h, and the paracellular flux was determined by measuring the fluorescence intensity at the basolateral compartment with a fluorometer (Promega Corp.) set at excitation/emission wavelengths of 410/520nm. After measurement, PBECs were harvested for immunofluorescence staining and western blotting.

ELISA measurement

Levels of IL-8 (Mabtech, Nacka Strand, Sweden), IL-6 (Invitrogen, ThermoFisher Scientific, Waltham, MA), IL-1β (Invitrogen) and TNF-α (R&D Systems, Minneapolis, MN) in the BALF and blood of calves were determined by using ELISA kits according to manufacturer's instructions. IL-8 (R&D Systems) and IL-6 (BioLegend, San Diego, CA) release in the supernatants of A549 or PBECs after different treatments was also measured by ELISA. The absorbance was measured at 450nm using a microplate reader (Bio-Rad, Hercules, CA).

Statistical analysis

Experimental results *in vivo* are expressed as non-transformed means ± SEM. *In vivo* data were analyzed for treatment and time effects with SAS 9.4 (SAS Institute Inc., Cary, NC), using the MIXED procedure, including time as a repeated statement with calf as unit. For each parameter, the covariance structure was selected based on the lowest AIC and BIC. All analyses included a random effect of pen. For blood leukocyte concentration, the concentration at arrival (before application of the treatments) was included as a co-variable in the model. Studentized residuals of each model were checked visually on the homogeneity

of variance and data were transformed if required to obtain homogeneity of variance. To evaluate differences between treatments, the contrast statement was used, and treatment differences were assessed per timepoint separately. Clinical scores were assessed for treatment and time effects using the GLIMMIX procedure with a multinomial distribution including a random pen effect and potential differences between the treatments were evaluated using the contrast statement per timepoint. The Chi-square test was performed for the proportion of different lung lesions and the positivity of *M. haemolytica* in calves. Differences were considered significant when $P < 0.05$ and considered a trend when $P < 0.10$.

Data from *in vitro* experiments are determined by one-way ANOVA or two-way ANOVA followed by Tukey with selected comparisons as a *post hoc* test. All *in vitro* results are expressed as means ± SEM and analyzed using the GraphPad Prism version 7.0 software (San Diego, CA). Results were considered statistically significant when $P < 0.05$ and considered a trend when $P < 0.10$.

Sources of support: This research was performed in the public-private partnership 'CarboKinetics' coordinated by the Carbohydrate Competence Center (CCC, www.cccresearch.nl). CarboKinetics is financed by participating industrial partners Agrifirm Innovation Center B.V., Cooperatie AVEBE U.A., DSM Food Specialties B.V., FrieslandCampina Nederland B.V., Nutrition Sciences N.V., VanDrie Holding N.V. and Sensus B.V., and allowances of The Netherlands Organisation for Scientific Research (NWO; No. ALWCC.2015.4). Research grant funding (No. 201608320245) was received from the China Scholarship Council for Y. Cai.

Author contributions

Y.C. and S.B. conceptualized the study; S.B., G.F., M.S.G., and W.J.J.G. advised on study design; Y.C. and M.S.G. directed experiments, analyzed data, performed statistical analysis; Y.C. wrote the manuscript, S.B. and G.F. edited the manuscript. Y.C., S.B., G.F., M.S.G., and W.J.J.G. gave final approval of the version to be published.

Supplementary information

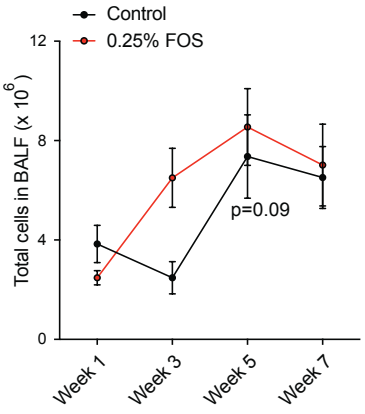

Figure S1. Effect of FOS on total cell numbers in BALF over time. Number of total cells in BALF was determined at experimental week 1, 3, 5 and 7 (n=40, 20 calves/group). *P*=0.09 (control week 5 vs week 1). Data are presented as means ± SEM.

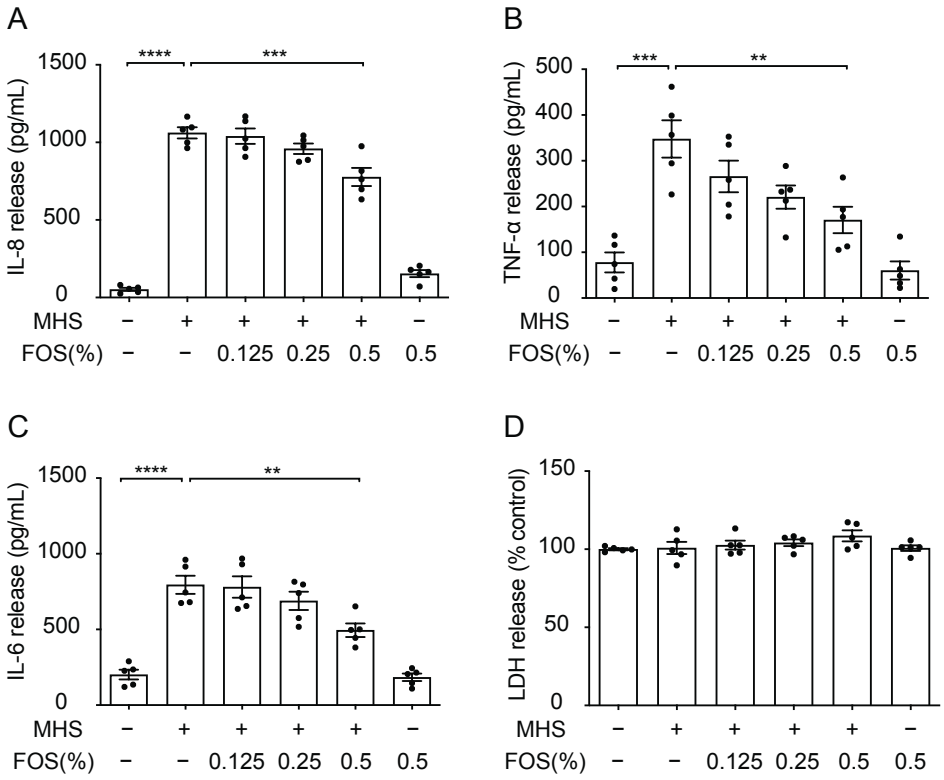

Figure S2. FOS suppress MHS-induced inflammation in PBECs. The supernatants containing virulence factors and other mediators were collected and filtered after *M. haemolytica* alone incubation for 24h and used as a stimulant for PBECs. Prior to the incubation with MHS (24h), PBECs were treated with or without FOS for 24h. (**A**-**C**) IL-8, TNF-α and IL-6 levels in the supernatants of PBECs were measured by ELISA. (**D**) LDH release was assessed in the supernatants of PBECs. ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of five independent experiments (n=5 donor calves).

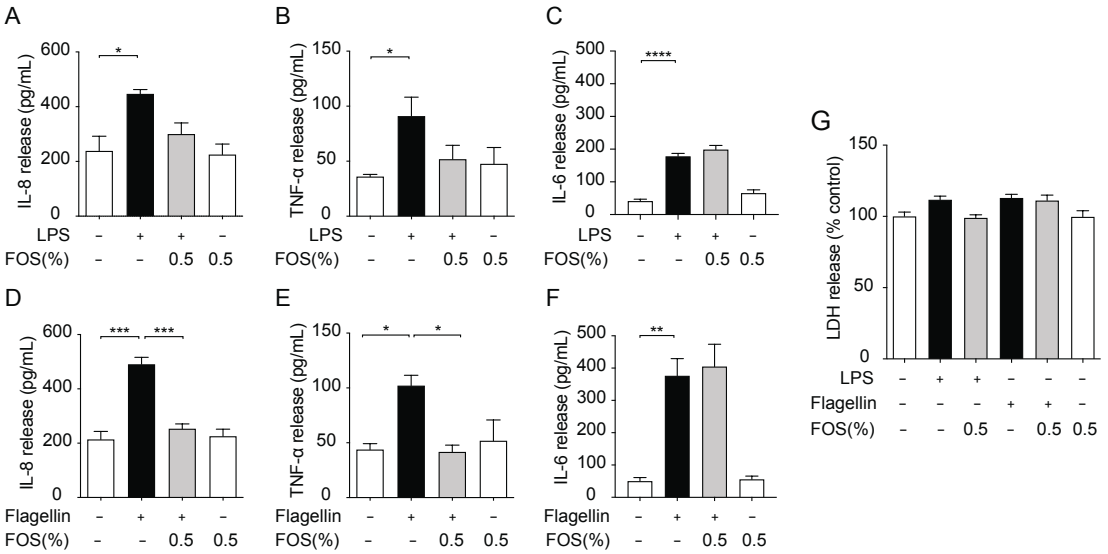

Figure S3. Anti-inflammatory effects of FOS in LPS- or flagellin-treated human alveolar epithelial cells. A549 cells were incubated with LPS (10 µg/mL) or flagellin (10 ng/mL) for 24h with or without 24h pretreatment with FOS. (**A**-**C**) IL-8, TNF-α and IL-6 levels in the supernatants of LPS-treated A549 cells were measured by ELISA. (**D**-**F**) IL-8, TNF-α and IL-6 levels in the supernatants of flagellin-treated A549 cells were determined by ELISA. (**G**) LDH release was assessed in the supernatants of A549 cells. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Data are presented as means ± SEM. All data shown are representative of four independent experiments (n=4).

Supplementary Methods

Animals and housing

Calves were housed in a mechanically ventilated stable throughout the experiment. The ambient lighting consisted of natural lighting plus artificial lighting from 0600 to 1800 h. Calves were housed in pens (9 m^2) containing wooden-slatted floors. In the first 6 weeks after arrival, individual housing was applied (1.2 m^2/calf) by placing stainless steel fences within the pens. After 6 weeks, the individual fencing was removed, and calves were housed in groups of 5.

Clinical scores

Clinical scoring was performed weekly for all calves, according to the Wisconsin calf respiratory scoring system [39], in which a score from 0 to 3 was provided for rectal temperature, coughing, nasal discharge and behavior. The point scale used for respiratory clinical scoring was used as we previously described (Chapter 3, Table 2). Clinical score was calculated as the sum of these 4 scores.

Blood sampling and hematological analyses

Blood samples were collected of all calves by venipuncture in the jugular vein at arrival before the first MR feeding (baseline, week 0), and additionally in experimental week 2, 4 and 6 from 20 calves per group. Blood was collected in 9 mL and 4 mL K_2-EDTA tubes and was kept on ice for collection of plasma or kept at room temperature for analysis of leukocyte numbers the same day by fluorescence flow cytometry using a Sysmex 1800iV (Sysmex Europe GmbH, Norderstedt, Germany), respectively. Plasma was collected after centrifugation at 2,000 x *g* and 4°C for 20 min and was stored at -20°C pending further analyses (cytokine/chemokine measurement by ELISA).

BALF sampling and phenotyping

Bronchoalveolar lavage fluid samples were obtained by use of a technique adapted from a previous description [40]. Briefly, a calf was restrained in the feeding fence and the head of calf was lifted and extended so that the nasal bone was parallel to the ground. 70% ethanol was used to clean the nose/nostrils of the calf. A sterilized 100 cm BAL catheter was then inserted through a naris and blindly guided through the nasal passage into the trachea until the end was wedged in a bronchus. The correct placement of the catheter was verified by elicitation of the coughing reflex, the outstretch of the tongue, movement of air into and out of the catheter with each breath, and the absence of rumen contents, odor, and gurgling from the catheter. Once wedged in the appropriate location, a syringe was connected to the catheter and a total of 30 mL of sterile saline (0.9% NaCl, 37°C) solution was infused into the tube and fluid was immediately aspirated from the bronchus. BALF (18.1 ± 0.39 mL) was obtained from each calf and was stored in a 50 mL tube on ice until further processing in the lab the same day.

After transport and arrival at the lab, the volume of BALF was recorded and BALF suspension was filtered by passing through a 70 µm cell strainer (Corning, NY, US) to remove debris. To obtain cell pellets and perform cell counts, BALF suspension was centrifuged (5 min, 400 x *g* at 4°C) and the remaining pellet was re-suspended in 1 mL cold FCS (4°C). After centrifugation, the supernatant was aliquoted into 1.5 mL tubes and stored at -80°C for further analysis. Cell number was determined by automatically counting in Cellometer Bright Field cell counter (Nexcelom Bioscience, Lawrence, MA). After counting, 0.5 x 10^6 cells of BALF suspension were used to make cytospins and phenotyping were determined by Diff-Quick (Medion Diagnostics, Medion Diagnostics International Inc., Miami, FL) staining on cytospin preparations and a minimum of 400 cells were counted.

LDH assay

PBECs or A549 cells were grown in 96-well plates as described above and the cytotoxic effect of LPS, flagellin, *M. haemolytica* or MHS on the PBECs or A549 cells was evaluated by measuring LDH leakage. LDH was measured in the supernatants using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega Corp.) according to manufacturer's instructions.

Western blotting

Cell lysates of PBECs in transwells after different treatments were prepared by adding RIPA cell lysis buffer (ThermoFisher Scientific) containing protease and phosphatase inhibitors (Roche, Penzberg, Germany). Total protein content was estimated by bicinchoninic acid analysis (ThermoFisher Scientific) according to the manufacturer's protocol. Samples were loaded onto polyacrylamide gradient gels (Criterion Gel, 4-20% Tris-HCl, Bio-Rad) and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with PBS containing 0.05% Tween-20 (PBST) and 5% milk proteins for 1h at room temperature and incubated with primary antibodies at 4 °C overnight (p-p38, 1:1000, p-ERK1/2, 1:1000, p-JNK1/2, 1:1000, p-p65, 1:1000, β-actin, 1:5000, Cell Signaling Technology, Beverly, MA), followed by washing blots in PBST. Appropriate horseradish peroxidase-coupled secondary antibodies from Dako (Agilent Technologies, Santa Clara, CA) were applied for 1h. Membranes were incubated with ECL western blotting substrates (Bio-Rad) prior to obtaining the digital images. Digital images were acquired with the Molecular Imager (Gel DocTM XR, Bio-Rad) and analyzed with Image lab 5.0 (Bio-Rad).

Immunofluorescence

PBECs were grown in transwells as described above and detected for the tight junction protein zonula occludens-1 (ZO-1) and the adherens junction protein E-cadherin using immunofluorescence. PBECs were fixed with 10% formalin (Baker, Deventer, The Netherlands) and after washing with PBS, the cells were permeabilized with PBS containing 0.1% (v/v) Triton-X-100 for 5 minutes, followed by blocking with 5% serum in 1% (w/v) BSA/PBS for 30 minutes at room temperature. Thereafter, PBECs were incubated overnight with primary antibodies ZO-1 (1:50, Abcam, Cambridge, UK) and E-cadherin (1:50, BD Biosciences, San Jose, CA) followed by incubation with Alexa-Fluor conjugated secondary antibodies (Invitrogen) for 1h at room temperature in dark. Nuclear counterstaining was performed with DAPI containing anti-fade reagent (ready to use, Invitrogen). ZO-1 and E-cadherin were visualized, and images were taken using the Keyence BZ-9000 (Osaka, Japan). Fluorescence intensity was quantified by Image J (Version 1.8.0, National Institutes of Health, US) and presented as fluorescence intensity (vs DAPI).

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Part III

Relevance to Human Respiratory Infections and Health

7

Chapter 7

Bacteriostatic Effect of Fructo-oligosaccharides and Bactericidal Effect of Galacto-oligosaccharides on *Mycoplasma Pneumoniae in vitro*

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(Submitted for publication)

Abstract

Background

Emerging antimicrobial resistance in *Mycoplasma pneumoniae* (MP), an important human respiratory pathogen, demands for novel intervention strategies. We investigated whether non-digestible oligosaccharides (NDOs), galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) with nutraceutical potential may inhibit the growth of MP *in vitro*.

Methods

MP and macrolide-resistant MP (MRMP) were cultured and the minimum inhibitory and bactericidal concentration (MIC/MBC) of GOS and FOS against MP and MRMP were determined. The kinetics of MP/MRMP growth in the presence and absence of GOS were monitored during 24h. The effects of GOS on P1 adhesin and 16S rRNA gene expression of MP/MRMP were studied by PCR. The potential additive effect of GOS/FOS and the antibiotic, erythromycin, against MRMP was investigated as a combination therapy.

Results

GOS and FOS inhibited the growth of MP and MRMP. The MIC of GOS for MP and MRMP was 4% and 2%, respectively. The MIC of FOS for both MP and MRMP was 16%. The observed decrease in P1 adhesin gene expression of MP and MRMP after GOS incubation might be due to the bactericidal effect of GOS. The MBC of GOS for MP and MRMP was 16%. GOS exhibited an additive effect on erythromycin at 64 μg/mL against MRMP.

Conclusion

This study describes for the first time that GOS and FOS can inhibit the growth of MP and MRMP, especially GOS have a bactericidal effect *in vitro*. Besides the prebiotic function, we showed a unique anti-bacterial role for NDOs against a leading pulmonary pathogen in children, which expands the potential therapeutic utility of these NDOs.

Introduction

Mycoplasma pneumoniae (MP) is one of the most common bacterial species causing community-acquired pneumonia (CAP) in children [1]. MP is one of the smallest free-living bacteria and it needs to obtain the critical nutrients from host cells due to its limited metabolic capability and small genome [2]. To obtain these nutrients, MP colonizes the respiratory tract by adhering to the epithelium through specialized terminal organelles containing adhesion proteins, such as P1 [3]. Despite most MP-induced CAP are mild, severe cases can also occur and require hospitalization and antibiotic treatments [4]. The extensive use of macrolides (e.g., erythromycin), the widely used and effective antibiotics against MP, has led to an alarming increase in the prevalence of macrolide-resistant MP (MRMP) strains in many parts of the world, predominantly in Asia [5]. The emergence of resistant bacteria highlights the importance of developing of antibiotic alternatives.

Non-digestible oligosaccharides (NDOs), including galacto-/fructo-oligosaccharides (GOS/FOS), are a group of dietary carbohydrates with potential anti-pathogenic functions [6, 7]. Children who received formula enriched with GOS/FOS have a decreased incidence and symptoms of respiratory infections [8, 9]. In addition to the well-known anti-adhesion properties [7], GOS can lower the number of bovine respiratory pathogens *in vitro* as well as *in vivo* via intranasal and dietary GOS application (Chapter 4 and Chapter 5). Similar anti-pathogenic functions have been described for chito-oligosaccharides (COS) and alginate-derived oligosaccharides. Both types of NDOs suppressed the growth and biofilm development of *Pseudomonas aeruginosa.* Furthermore, COS also exhibited synergistic effects with azithromycin (one of the macrolides) against a multi-drug resistant *P. aeruginosa* strain *in vitro* [10].

Here, we hypothesize that FOS and GOS may affect the growth of MP and the anti-bacterial efficacy of erythromycin *in vitro*. We showed for the first time that GOS have the capacity to kill (the vast majority of) MP and MRMP *in vitro,* while FOS only inhibited growth. Interestingly, the efficacy of erythromycin against MRMP was increased by GOS supplementation. NDOs, such as GOS and FOS, with or without the combination of standard drugs might be potential strategies for the prevention and treatment of MP-related respiratory infections.

Results

GOS and FOS inhibit the growth of MP and MRMP

To test whether GOS and FOS can inhibit the growth of MP and MRMP, minimum inhibitory concentration (MIC) assays were performed. Therefore, MP and MRMP were incubated with increasing concentrations of GOS or FOS, or with erythromycin as a positive control. After 5-6d incubation, the MIC of erythromycin for MP was 0.032 µg/mL, while the MIC values of GOS and FOS were 4% and 16%, respectively (**Table 1**). The MIC of erythromycin for the MRMP strain was 256 µg/mL, 8,000-fold higher than for the macrolide-sensitive MP (**Table 1**). However, the MIC values of GOS and FOS for MRMP were similar as for MP, being 2% and 16%, respectively (**Table 1**).

Table 1. The minimum inhibitory concentration (MIC) of erythromycin, GOS and FOS for MP and MRMP.

	MIC for:		
Strains	Erythromycin	GOS	FOS
MP	0.032 µg/mL	4%	16%
MRMP	256 µg/mL	2%	16%

MP, *Mycoplasma pneumoniae*; MRMP, macrolide-resistant *Mycoplasma pneumoniae*; GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides.

GOS exhibit the capacity to reduce the viability of MP and MRMP

To determine whether the effect of GOS and FOS depicted in **Table 1** was bacteriostatic or bactericidal, supernatants from GOS, FOS or erythromycin-treated MP/MRMP were inoculated onto SP4 agar plates to evaluate the minimum bactericidal concentration (MBC). Untreated MRMP and MP yielded on average 0.28 x 10^8 and 3.22 x 10^8 CFU/mL, respectively (**Figure 1A** and **D**). Subcultures of erythromycin at concentrations of 256 and 512 µg/mL confirmed the resistance of MRMP to erythromycin as these yielded lower amounts of MRMP CFUs than those of untreated controls (**Figure 1A**), while higher than the initial inoculum (red dotted line). In addition, the vehicle control (i.e., 2.58% ethanol) did not affect the growth of MRMP CFUs. Moreover, subcultures of MP treated with erythromycin at concentrations of 0.032 µg/mL indicated the bactericidal effect of erythromycin on MP as shown by the lower of MP CFUs than the initial inoculum (**Figure 1D**).

Although 16% FOS seemed to inhibit the growth of MRMP (**Table 1**), FOS did not affect the recovery of MRMP CFUs on agar plates (**Figure 1B**). In contrast, 8% GOS lowered CFUs of MRMP by 99.8% than controls. Furthermore, 16% GOS completely killed MRMP as shown by no visible growth of MRMP CFUs (**Figure 1C**). As expected, GOS at concentrations of 16% also reduced the viability of MP as shown by the lower of MP CFUs than the initial inoculum (**Figure 1D**). Overall, FOS did not exhibit bactericidal behavior, while 16% GOS killed the MRMP strain and the vast majority (98%) of MP strain.

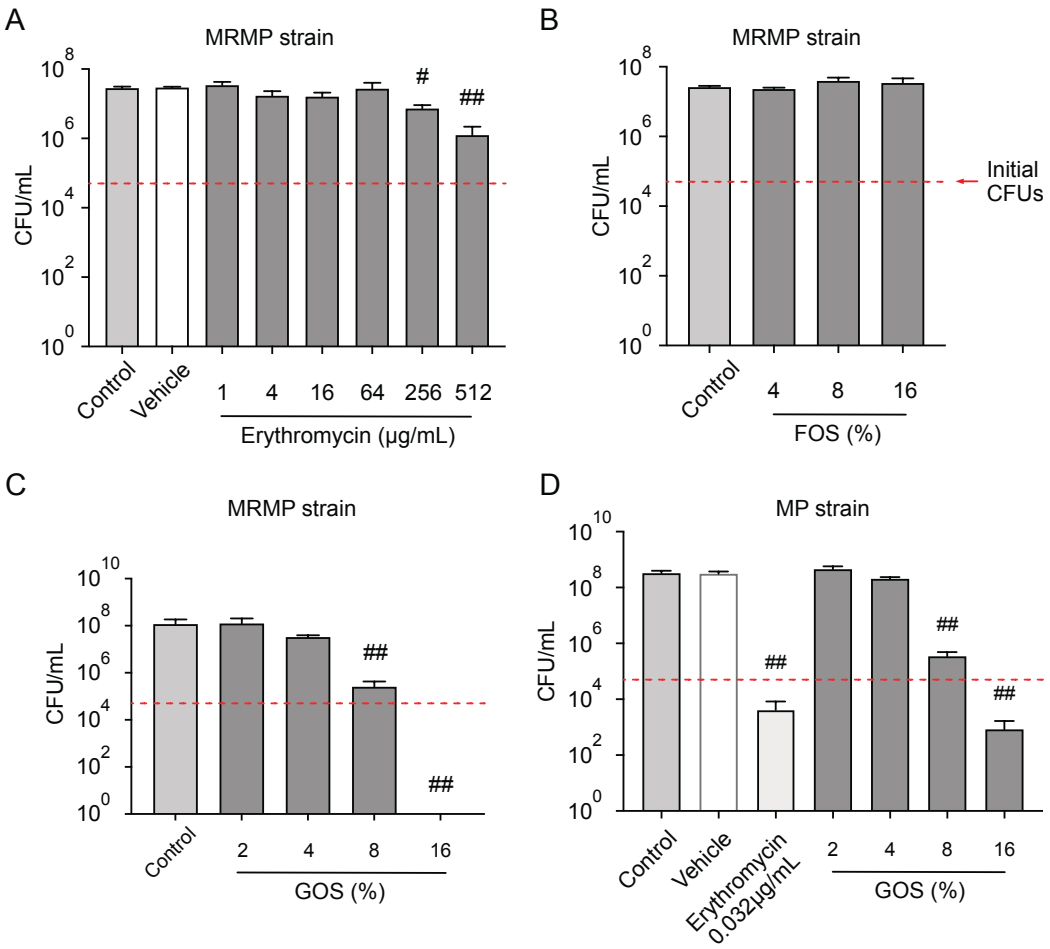

Figure 1. Effect of erythromycin, FOS and GOS on the growth of MP/MRMP. MP and MRMP were incubated with or without different concentrations of erythromycin, GOS or FOS for 5-6d, thereafter supernatants were diluted and inoculated onto SP4 agar plates. (**A-D**) MRMP or MP numbers were determined by counting CFUs on each agar plate and calculated as CFU/mL based on the dilution factor. The red dotted line represents the CFUs of the initial inoculum. #*P*<0.05; ##*P*<0.01 (erythromycin, GOS, or FOS treatments vs control group). Data are presented as means ± SEM. All data shown are representative of three independent experiments (n=3).

GOS exhibit bactericidal activity within 24h

To investigate the kinetics of anti-bacterial activity of GOS, 0.4 x 10^5 CFU/mL MP and MRMP were incubated with or without GOS for 6 and 24h. Thereafter, the supernatants were inoculated onto SP4 agar plates to determine the MBC. As shown in **Figure 2**, the CFUs of untreated MP and MRMP after 6h incubation were less than or close to the initial CFUs present at t=0. However, CFUs of untreated MP and MRMP increased within 24h as compared with t=0 (**Figure 2**). Moreover, the CFUs of 16% GOS-treated MP and 8% and 16% GOS-treated MRMP were already significantly reduced within 24h compared to untreated MP and MRMP. Compared with the CFUs of initial inoculation, less growth or even death of MP and MRMP was observed with 16% GOS (**Figure 2**). Overall, GOS have bactericidal activity within 24h.

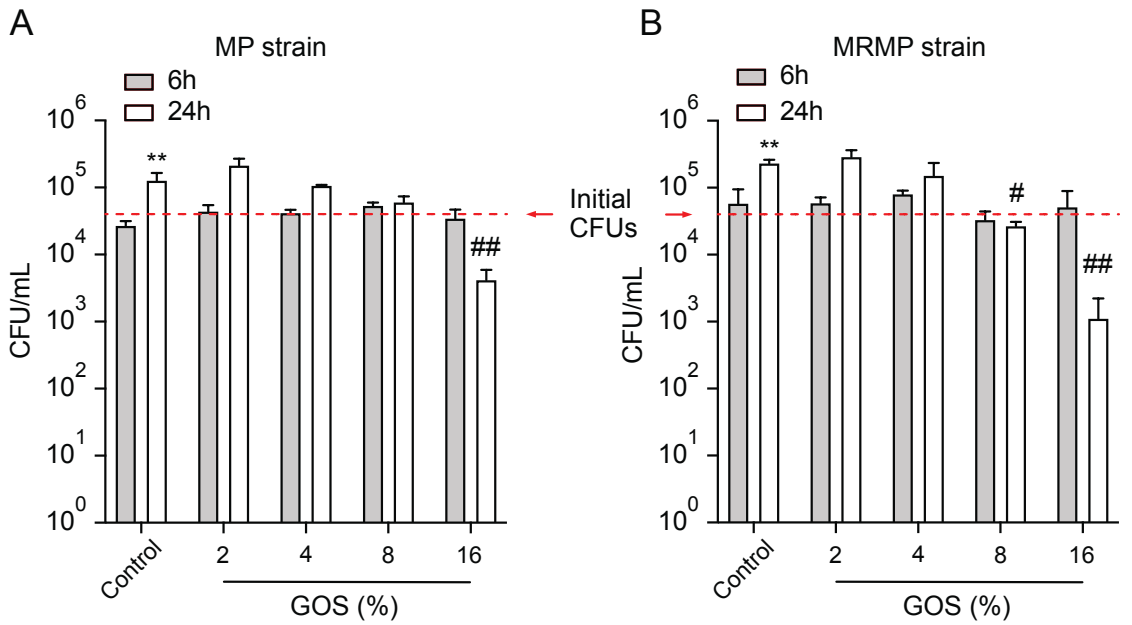

Figure 2. GOS exhibit bactericidal activity within 24h. MP and MRMP was incubated with or without increasing GOS concentrations for 6 and 24h, thereafter supernatants were diluted and inoculated onto SP4 agar plates. (**A-B**) MP and MRMP numbers were determined by counting CFUs on each agar plate and calculated as CFU/mL based on the dilution factor. The red dotted line represents the CFUs of the initial inoculum. ***P*<0.01 (control 24h vs 6h); #*P*<0.05, ##*P*<0.01 (24h GOS treatments vs 24h control group). Data are presented as means ± SEM. All data shown are representative of three independent experiments (n=3).

Decreased expression of P1 adhesin gene is related to the bactericidal activity of GOS

The adhesin molecule P1 is responsible for the adhesion of MP to host cells [1, 2]. Previous studies indicate that in addition to the anti-pathogenic effects, GOS can also prevent the adhesion of pathogens to host epithelial cells [6, 11]. Therefore, the P1 adhesin gene expression in MP and MRMP strains was investigated after 5-6d incubation in presence and absence of GOS. Increased Cq values were observed in MP and MRMP incubated with GOS concentrations ≥8%, indicating that GOS inhibited expression of P1 adhesin gene (**Figure 3**).

To investigate whether the decreased P1 adhesin gene was a direct effect of GOS or a reflection of the reduced bacterial numbers, the expression levels of the 16S rRNA gene of MP were used as an internal control. As shown in **Figure 3**, the expression levels of P1 adhesin and 16S rRNA genes of MP and MRMP are synchronous with high Cq levels in MP and MRMP incubated with GOS, indicating that the decreased expression of the P1 adhesin gene is related to the reduced bacterial numbers. Additionally, the 16S rRNA gene data in **Figure 3B** are confirming the bactericidal effect of GOS against MP and MRMP (MBC assay) as depicted in **Figure 1C** and **D**.

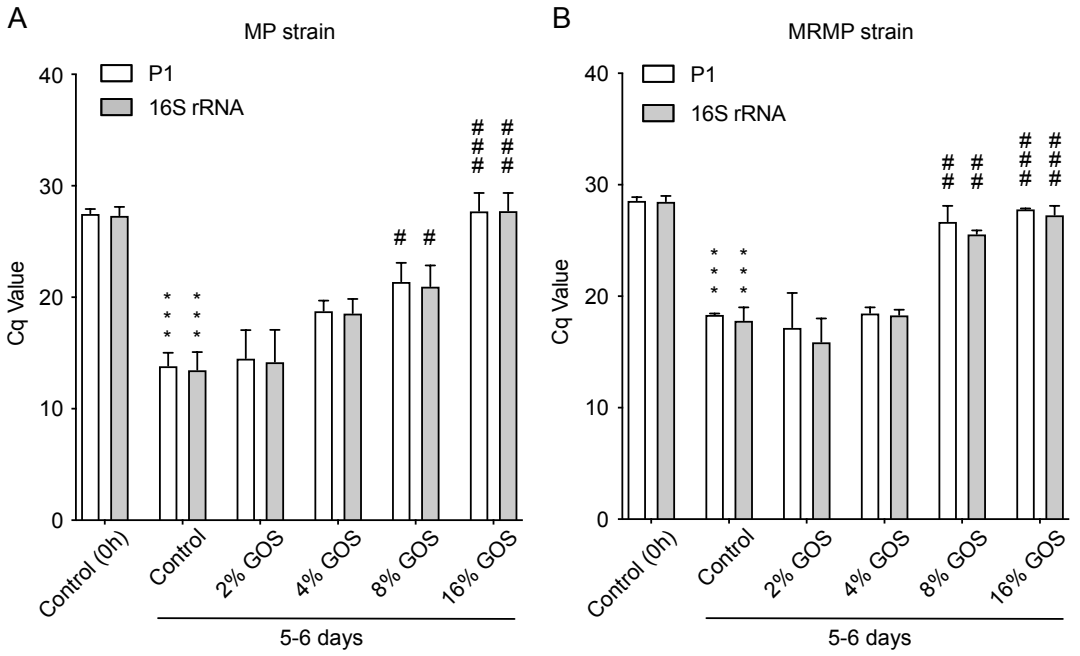

Figure 3. Decreased expression of P1 adhesin gene is related to the bactericidal activity of GOS. MP and MRMP were incubated with or without increasing concentrations of GOS for 5-6d, thereafter P1 and 16S rRNA genes of MP and MRMP were investigated by real-time PCR. ***P<0.001 (control 5-6d vs 0h); ##P<0.05, ##P<0.01, ###P<0.001 (GOS treatments vs control 5-6d). Data are presented as means ± SEM. All data shown are representative of two independent experiments (n=2).

Differential effects of GOS and FOS on the resistance of MRMP to erythromycin

To investigate whether GOS or FOS has an additive effect on the anti-bacterial efficacy of erythromycin against MRMP, supernatants were inoculated onto SP4 agar plates after incubation of MRMP with erythromycin alone, or in combination with GOS or FOS for 5-6d. As shown in **Figure 4A**, FOS did not exhibit an additive effect on the anti-bacterial efficacy of erythromycin at 4 and 16 μg/mL, while even reducing the effectivity of erythromycin at 64 μg/mL. In contrast, 4% GOS showed an additive effect on the anti-bacterial efficacy of erythromycin at 64 μg/mL, since a higher anti-bacterial efficacy (78%) was observed in this combination (4% GOS + 64 μg/mL erythromycin)-treated MRMP than control (0%), 4% GOS (47%) and 64 μg/mL erythromycin (27%) groups (**Figure 4C**). In addition, no or few (less than the initial inoculum) visible CFUs were observed when 16% GOS in combination with erythromycin from 4 to 64 μg/mL, while this is mainly due to the

bactericidal effect of 16% GOS (**Figure 4B**). Moreover, no visible CFUs of MRMP were observed in the treatment of 8% GOS + 64 µg/mL erythromycin, while 1.5 x 10^4 and 2.7 x 10^7 CFU/mL of MRMP were counted after 8% GOS and 64 µg/mL erythromycin alone treatment (**Figure 4B**), respectively.

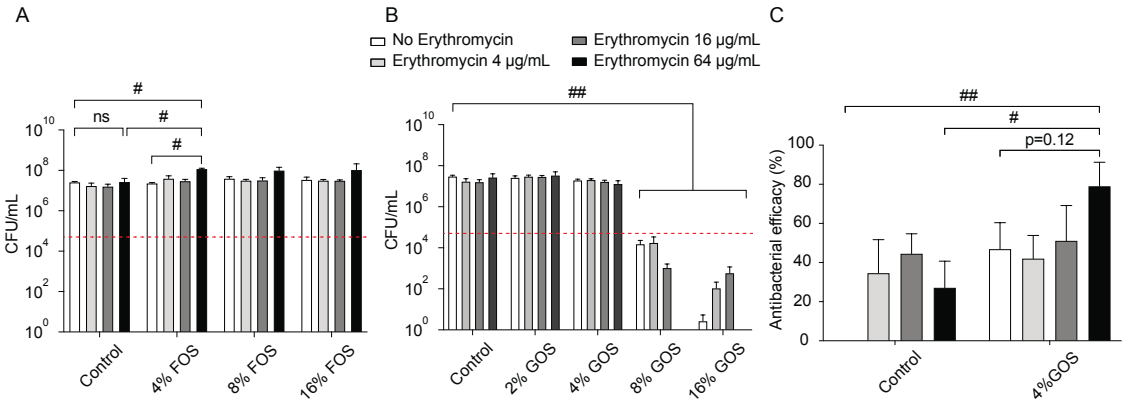

Figure 4. Differential effects of GOS and FOS on the resistance of MRMP to erythromycin. MRMP was incubated with erythromycin or with the combination of GOS or FOS and erythromycin for 5-6d, thereafter supernatants were diluted and inoculated onto SP4 agar plates. (**A-B**) MRMP number was determined by counting CFUs on each agar plate and calculated as CFU/mL based on the dilution factor. (**C**) Anti-bacterial efficacy was calculated based on the CFUs and shown as percentages. The red dotted line represents the CFUs of the initial inoculum. #*P*<0.05; ##*P*<0.01. Data are presented as means ± SEM. All data shown are representative of at least three independent experiments (n=3 or 4).

Discussion

Here, we demonstrated for the first time that GOS can (partly) kill both macrolide-sensitive and resistant MP *in vitro*, while FOS only lowered the growth of these MP strains. In addition, GOS showed a slight additive effect on the anti-bacterial efficacy of erythromycin at 64 µg/mL against MRMP.

NDOs are well known for their prebiotic activity and the modulation on the gut microbiota composition [6, 12]. NDOs can exert immunomodulatory effects dependent, but also independent of intestinal microbiota, influencing human and animal health [6]. A variety of protective properties in infections and inflammations have been described for NDOs [6, 11]. In a previous animal (calf) study, we demonstrated that GOS can strongly lower the viability of bovine respiratory pathogen, *Mannheimia haemolytica* (Gram-negative opportunistic bacteria) and

reduce the adhesion to and invasion of bronchial epithelial cells by *M. haemolytica* (Chapter 5). Interestingly, we now found that both GOS and FOS can inhibit the growth of the human respiratory pathogen *M. pneumoniae* (macrolide-sensitive and resistant strains), while GOS showed unique bactericidal effects on both MP strains within 24h.

The absence of a cell wall and only the presence of a plasma membrane makes MP unique among all bacteria and exhibit many peculiarities, including morphological instability, osmotic sensitivity, special ion pumping systems, resistance to antibiotics that interfere with bacterial cell wall synthesis, such as β-lactams [13]. NDOs can interfere with the bacterial membrane and herewith affect the metabolism of bacteria [6, 14, 15]. Moreover, due to the increased membrane permeability of group B *streptococcus* by HMOs, the anti-bacterial functions of aminoglycosides, lincosamides, macrolides, and tetracyclines but not β-lactams can be potentiated [14]. In addition, the blockage of nutrient flow caused by aggregation of COS and negatively charged O-specific antigenic oligosaccharide-repeating units of the outer bacterial membrane was the main cause of growth inhibition and lysis of *Escherichia coli* caused by COS [15]. In the present study, we showed that GOS killed both MP strains and increased the anti-bacterial efficacy of erythromycin against MRMP, which might be related to an increased membrane permeability of MP/MRMP induced by GOS, possibly leading to blockage of the nutrient intake. This possibility is in line with observations from our *in vitro* study, in which GOS reduced viability of *M. haemolytica,* possibly by increasing membrane permeability (Chapter 5). To confirm the results obtained with the MBC assay (Figure 4), measurement of P1 and/or 16S rRNA gene expression in MRMP treated with the combination GOS and erythromycin might be useful.

The anti-bacterial efficacy of GOS was higher as compared to FOS. 2% GOS lowered the growth of MRMP and 16% GOS killed MRMP, while 16% FOS was required to lower the growth of MRMP. NDOs with distinct chemical structures exert different functions in the modulation of microbial growth [6, 7]. In an *in vitro* study, pectic oligosaccharides completely inhibited the growth of *Clostridium perfringens* and *Bacteroides fragilis* strains, whereas FOS enhanced their growth within 24h [16]. Moreover, GOS were shown to inhibit but COS to increase the growth of *E. coli*, while FOS had no significant effect on the growth of *E. coli* [17]. In our previous studies, 4% GOS lowered the growth of *M. haemolytica* and 16% GOS killed the vast majority of *M. haemolytica in vitro* (Chapter 5), while 1% FOS increased the growth of *M. haemolytica* within 24h (Chapter 6). In the present study, we observed that the combination of FOS and 64 μg/mL erythromycin even promoted the subsequent growth of MRMP on the agar plate, which is difficult to

explain. Drug interactions are subtle in drug-resistant pathogens and can even promote the existence of resistant strains when drugs degrade to non-antibiotic metabolites [18].

NDOs (e.g., GOS) can act as soluble decoy receptors to prevent the adhesion of pathogens to epithelial surfaces [6, 11]. The adherence of enteropathogenic *E. coli, Cronobacter sakazakii,* and *Citrobacter rodentium* to host epithelial cells are inhibited by GOS [19-21]. Breastfeeding infants ingest mother milk several times per day, bathing the nasopharynx for several minutes at each feeding with a solution high in HMOs, which might inhibit respiratory bacterial adherence [22]. Our previous study in calves also showed that intranasal GOS administration might limit the invasion of opportunistic bacteria (e.g., *Pasteurellaceae*) from the upper respiratory tract into the lower respiratory tract (Chapter 5), while the *ex vivo* results pointed out that GOS inhibit adhesion to and invasion of bronchial epithelial cells by *M. haemolytica* (Chapter 5).

P1 adhesin is a major protein associated with the adherence of MP to host cells of the respiratory tract and responsible for binding to sialic acid oligosaccharide receptors from the host cells [23, 24]. Therefore, there is a possibility that GOS act as a decoy receptor to competitively bind to these oligosaccharide receptors of host cells, thereby inhibiting the adhesion of P1. In the current study, GOS inhibited the gene expression of P1 adhesin in MP/MRMP, although this is more likely to be associated with the decreased bacterial numbers induced by GOS supplementation as reflected by reduced 16S rRNA expression levels.

Future animal and clinical studies may help to identify functional associations of NDOs, like GOS, with the severity of MP/MRMP infections. NDOs with or without the combination of standard drugs might be helpful in the prevention or treatment of human (childhood) respiratory infections.

Materials and Methods

Bacteria

Macrolide-resistant *M. pneumoniae* A58 strain (clinical isolate derived from symptomatic CAP patient as described before) [25] and *M. pneumoniae* M129 strain (subtype 1; ATCC 29342) were cultured in SP4 medium as previously described [26].

Determination of minimum inhibitory concentration (MIC)

MRMP A58 strain (50 µL at 2 x 10^5 CFU/mL) and MP M129 strain (50 µL at 2 x 10^5 CFU/mL) were inoculated to 96-well sterile polystyrene plates in the absence or presence of increasing concentrations of erythromycin, GOS or FOS to achieve a final volume of 250 µL per well. The final concentrations of erythromycin in MRMP and MP ranging from 1 to 1024 µg/mL and from 0.001 to 1.024 µg/mL, respectively. The final concentrations of GOS or FOS in cultures with MRMP or MP ranging from 0.5% to 16%. The plates were incubated under static conditions at 37°C/5% CO_2 for 5-6d. Bacterial growth was quantified through identifying the color of the medium. The MIC was assigned at the lowest concentration of compound at which no change in medium color was observed.

Determination of minimum bactericidal concentration (MBC)

MP and MRMP was incubated with or without increasing concentrations of erythromycin, GOS or FOS for 5-6d as described above. Additionally, MRMP was incubated with the negative control, 2.58% ethanol (vehicle of erythromycin). The supernatants (20 µL) were 1,000-fold diluted and inoculated onto SP4 agar plates for 10d at 37°C/5% CO_2. Thereafter, CFUs of MRMP were visualized by the addition of 500µL of 600 mg/L iodonitrotetrazolium chloride and counted with a microscope.

Anti-bacterial activity of GOS within 24h

MP and MRMP was incubated with or without increasing concentrations of GOS for 6 and 24h as described above. The supernatants (20 µL) were 10-fold diluted and inoculated onto SP4 agar plates for 10d at 37°C/5% CO_2. CFUs of MP were visualized by the addition of 500µL of 600 mg/L iodonitrotetrazolium chloride and counted with a microscope.

Real-time PCR

DNA isolation was performed on 200 µL of the supernatants using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A quantitative TaqMan (Applied Biosystems, Foster City, CA) real-

time PCR assay was used to detect and quantify cytadhesin P1 and 16S rRNA levels of *M. pneumoniae*, as previously described [25, 27, 28].

MBC assay of the combination of GOS/FOS and erythromycin

MRMP was incubated with increasing concentrations of GOS/FOS and erythromycin or with the combination of GOS/FOS and erythromycin for 5-6d as described above, thereafter supernatants were 1,000-fold diluted and inoculated onto SP4 agar plates for 10d at 37°C/5% CO_2. After that, CFUs of MP were visualized by the addition of 500 µL of 600 mg/L iodonitrotetrazolium chloride and counted with a microscope.

Anti-bacterial efficacy was calculated based on the control CFUs (a) and treatment CFUs (b) using (1-b/a) and expressed as percentage.

Statistical analysis

Statistically significant differences between groups were determined by one-way ANOVA or two-way ANOVA with post hoc Dunnett's multiple comparisons test using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA). Results were considered statistically significant when $P < 0.05$.

Author contributions: Y.C., S.B., and W.W.J.U. conceptualized the study; S.B., G.F., and W.W.J.U. advised on study design; Y.C., L.S., and H.Z. directed experiments, analyzed data, performed statistical analysis; Y.C. wrote the manuscript, S.B., G.F., and W.W.J.U. edited the manuscript. Y.C., L.S., H.Z., G.F., S.B., and W.W.J.U. gave final approval of the version to be published.

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8

Chapter 8

Microbiota-dependent and -independent Effects of Dietary Fiber on Human Health

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This chapter is published in *British Journal of Pharmacology*, 2020. 177(6): p.1363-1381.

Abstract

Dietary fiber, such as indigestible oligo- and polysaccharides, occurs in many foods and has gained considerable interest related to its beneficial effects on host health and specific diseases. Dietary fiber is neither digested nor absorbed in the small intestine and modulates the composition of the gut microbiota. New evidence indicates that dietary fiber also directly interacts with the epithelium and immune cells throughout the gastrointestinal tract by microbiota-independent effects. This review provides a focused overview of how dietary fiber improve human health and how these reported health benefits are connected to molecular pathways, in (1) a microbiota-independent manner, via interaction with specific surface receptors on epithelial and immune cells regulating intestinal barrier and immune function, (2) a microbiota-dependent manner via maintaining intestinal homeostasis by promoting beneficial microbes, including *Bifidobacteria* and *Lactobacilli*, limiting the growth, adhesion and cytotoxicity of pathogenic microbes, as well as stimulating fiber-derived microbial short-chain fatty acid production.

Keywords: Dietary fiber; Microbiota; Immune system; Gut homeostasis; SCFAs

Introduction

The CODEX Alimentarius Commission defined dietary fiber as a group of carbohydrate polymer compounds with ten or more monomeric units (the footnote allows to include polymers with DP 3–9), which are neither digested nor absorbed in the human small intestine [1]. Food products that naturally contain dietary fiber, such as cereals, fruits, vegetables, nuts, beans and seafood, but also breast milk and application of prebiotics in functional food are main sources of dietary fiber intake. Some dietary fiber affect the digestion rate by reducing gastric emptying, limiting digestive enzyme activity, and by restricting the rate and extent of nutrient absorption in the gut. After ingestion, dietary fiber passes through the esophagus and stomach and reaches the small intestine followed by fermentation by the microbiota in the cecum and colon and, finally, absorption by the host [2].

The human gut microbiome, i.e., the microbiota that populate the human gastrointestinal (GI) tract, plays a significant role in maintaining gut homeostasis, including the digestion of dietary fiber, production of nutrients, vitamins and hormones, protection against pathogens and maintaining immune homeostasis [3]. Dietary habits are crucial for modulating the composition and function of the gut microbiome. Various types of dietary fiber have received considerable interest related to their microbiota-dependent effects on host health and certain diseases [4]. The composition of the gut microbiome, including the presence of *Bifidobacterium* and *Lactobacillus* species, is important for health-promoting effects, such as anti-allergic and anti-inflammatory properties [5]. The main end-products of intestinal bacterial fermentation of dietary fiber, the short-chain fatty acids (SCFAs), particularly acetate, propionate and butyrate, are regulators of gut homeostasis. These fiber-induced microbial changes and microbial fermentation products have a considerable impact on the immune system and hence on the prevention and treatment of diseases [6].

In addition to its microbiota-dependent effects, some dietary fiber interact with several cell types and protects intestinal epithelial barrier function, has immunomodulatory properties and can inhibit inflammation independent of the microbiota.

Here, we provide a detailed review of direct, i.e., microbiota-independent, effects of dietary fiber (**Figure 1**) on intestinal barrier function and immunity as well as relevant mechanisms. We also discuss the microbiota-dependent effects of dietary fiber on intestinal commensal and pathogenic microbiota as well as fiber-induced SCFA production and how these microbiota-dependent and -independent effects relate to the modulation of gut homeostasis in health and disease.

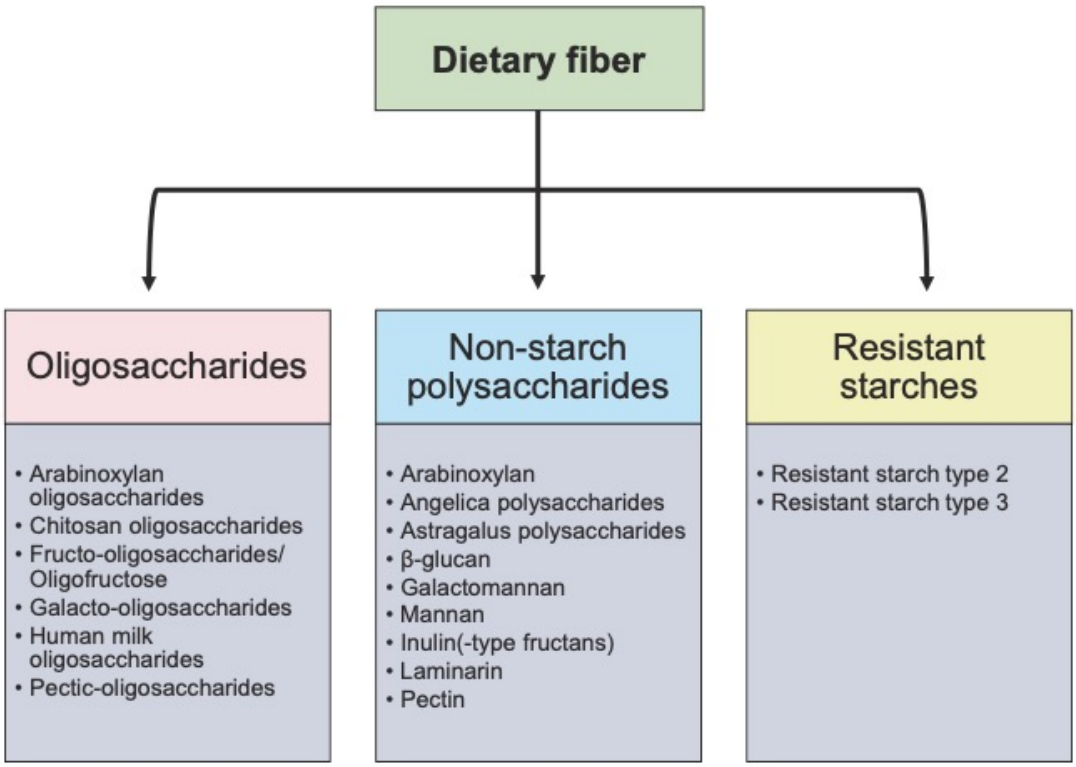

Figure 1. Groups of dietary fiber discussed in this review. Dietary fiber is usually categorized based on their chemical structure.

Microbiota-independent effects of dietary fiber

The intestinal mucosa is continuously exposed to food components as well as pathogenic and commensal microbes. This requires a homeostatic balance that allows for tolerating food antigens as well as beneficial, non-pathogenic commensal microbes, while defending against pathogenic microbes. The disruption of this homeostatic balance has been associated with the development of multiple (inflammatory) diseases, such as inflammatory bowel disease (IBD). To maintain intestinal homeostasis, a close interaction between the luminal microbiota, intestinal epithelium and immune cells is required [7].

Intestinal epithelial cells (IECs), which are responsible for the largest mucosal surface of the human body, are in direct contact with the external environment. Their main functions are the digestion of food and the absorption of nutrients, but they also act as a physical and biochemical barrier, preventing potentially

harmful antigens from invading the body. The luminal secretion of mucins by goblet cells provides the first protection against luminal antigens. Moreover, IECs are sealed by junctional complexes, such as tight and adherens junctions, which are important regulators of epithelial barrier function. Disruption of these junctional complexes leads to increased epithelial permeability (also known as a “leaky gut”) and predisposes to or enhances acute and chronic disease progression [8]. Related to the continuous exposure to pathogens, antigens and toxins, the intestinal epithelium shows rapid self-renewal in the event of tissue damage [9].

Innate as well as adaptive immune cells including dendritic cells (DCs), macrophages and mast cells (MCs) are influenced by IEC-derived signals (e.g., cytokines and chemokines, direct cell-cell contact) [7]. DCs efficiently acquire antigens from the intestinal lumen and can induce tolerance and immunity [10]. Macrophages maintain the intestinal homeostasis and are important components of protective immunity with high phagocytic activity [11]. Mast cells contribute to host defense against invading pathogens by regulating epithelial function and integrity and by raising innate and adaptive mucosal immune responses [12].

Dietary fiber passes the upper GI tract before reaching the large intestine to be fermented and interacts with IECs and immune cells along its intestinal journey. The direct interaction of fiber with different intestinal cell types promotes gut homeostasis and intestinal epithelial barrier function, and it supports intestinal immune responses, via effects on DCs, macrophages and MCs.

The effect of dietary fiber on intestinal epithelial barrier function

Several studies have highlighted the importance of dietary fiber in protecting intestinal barrier integrity. The human milk oligosaccharide (HMO), Lacto-Nneotetraose (LNnT), induced an increase in transepithelial electrical resistance (TEER) in Caco-2Bbe cells [13]. Galacto-oligosaccharides (GOS) mitigated the harmful effects of the mycotoxin deoxynivalenol on the intestinal barrier of Caco-2 cells, accelerating tight junction reassembly and stabilizing the expression and cellular distribution of the tight junction protein, claudin-3 [14]. In addition, GOS enhanced the mucosal barrier function via direct modulation of goblet cell function by upregulating gene and protein expression levels of secretory products and Golgi-sulfotransferases [15]. Astragalus polysaccharide (traditional Chinese medicinal herb) prevented the decreased occludin mRNA expression in lipopolysaccharides (LPS)-challenged Caco-2 cells [16], and a product rich in β-

galactomannan protected the S. *Enteritidis*-infected Caco-2 intestinal epithelial barrier and recovered the zonula occludens (ZO)-1 and occludin localization [17].

The epithelial glycocalyx is important for bacterial–epithelial cell interactions and can support microbial colonization and gut barrier function. 2'-fucosyllactose (2'FL), 3'FL and inulins were able to stimulate intestinal epithelial glycocalyx development, while pectins were less effective [18].

Dietary fiber also promotes intestinal barrier function by direct effects on the proliferation, migration, differentiation, and apoptosis of IECs. For example, HMOs induced apoptosis and inhibited proliferation in IECs by promoting arrest in the G2/M phase [19], and Holscher *et al*. indicated that there is an association between the inhibition in cell proliferation and an increase in cellular differentiation [13, 20]. High temperature-modified citrus pectins repressed the proliferation of G2/M cell-cycle arrest and induced apoptosis in a caspase-3-dependent way in different cancer cell lines [21]. In contrast, arabinoxylan and mixed-linked beta-glucans had no effect on the proliferation of Caco-2 and HT-29 cells [22].

In summary, different dietary fiber can protect intestinal barrier function via modulation of epithelial tight junction proteins and goblet cell function and possibly via regulation of epithelial cell and glycocalyx maturation.

Potential molecular mechanisms of the regulation of intestinal epithelial barrier function by dietary fiber

Activation of AMP-activated protein kinase by dietary fiber

AMP-activated protein kinase (AMPK) is an important regulator of IECs including their protective barrier function and tight junction assembly [23]. Chitosan oligosaccharides (COS) promoted the assembly of tight junction proteins in an IEC line by activation of AMPK via calcium-sensing receptor (CaSR)-phospholipase C (PLC)-IP_3 receptor channel-mediated calcium release from endoplasmic reticulum [24]. There is also a potential role for Toll-like receptors 2 (TLR2) in regulating intestinal epithelial barrier function, since activation of TLR2 by inulin-type fructans (ITF) and lemon pectins promoted the intestinal epithelial integrity, probably via a TLR2/MyD88/NF-κB signaling pathway [25, 26]. In addition, TLR2 signaling is involved in epithelial proliferation and apoptosis of terminally differentiated epithelial cells [27]. There are even indications that TLR2 signaling promotes mucin production [28, 29].

Dietary fiber interaction with the epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) can induce IEC proliferation and differentiation [30]. Neutral and acidic HMOs caused IEC cycle arrest by interacting with the EGFR as measured by EGFR phosphorylation. These oligosaccharides activated EGFR downstream signaling pathways, such as p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) 1/2 [19].

Taken together, different dietary fiber contains intestinal barrier protective properties by recovering the junctional network, modulating goblet cell function and possibly by increasing epithelial cell differentiation and glycocalyx maturation which is possibly related to direct interactions with AMPK, TLRs and EGFR. In addition, these types of dietary fiber modulate cytokine production and release by IECs and other cell types, thereby indirectly modifying intestinal barrier function.

The effect of dietary fiber on intestinal epithelial immunity

Dietary fiber can promote intestinal immunity by direct effects on IECs and on cells of the intestinal immune system including DCs, macrophages and MCs.

Immunological effects of dietary fiber that are mediated by IECs

Several *in vitro* studies have described the effects of dietary fiber on intestinal epithelial-derived cytokine and chemokine production and release using different IEC lines. Inulin, GOS and fructo-oligosaccharides (FOS) increased human growth-related oncogene-alpha (GRO-α), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2 and IL-8 secretion in some types of IECs [31], whereas α3-sialyllactose and chicory FOS inhibited baseline IL-12 release in Caco-2 cells [32]. In contrast, arabinoxylan and mixed-linked beta-glucans, did not affect the basal production of IL-8 by Caco-2 and HT-29 cells [22], which was in agreement with the unaltered basal expression of proinflammatory mediators in human colonic epithelial cells incubated with pooled HMOs [33].

The inhibition of cytokine/chemokine expression and release by several types of dietary fiber, including HMOs [33-35], GOS [14], arabinoxylan hydrolysates [36], arabinoxylans, mannose and galactomannans [37] was described in *in vitro* studies using different IECs exposed to inflammatory triggers. For example, HMOs and galactosyllactoses inhibit TNF-α-, *Salmonella*- and *Listeria*-induced IL-8, MIP-3α expression and release in T84, NCM-460 and H4 cells. The HMOs, 6'-sialyllactose (6'SL), can decrease IL-8 and MIP-3α release in T84 and HT-29

cells stimulated with antigen–antibody complex [34], whereas 2'-FL is effective in inhibiting the LPS-dependent induction of IL-8 in T84 cells [35]. HMOs from colostrum are able to attenuate PAMP-induced inflammatory cytokine protein levels, including IL-8, IL-6, MCP-1/2 and IL-1β [38]. In addition, GOS suppress the secretion of IL-8 in Caco-2 cells stimulated with the mycotoxin deoxynivalenol, while FOS and inulin are not effective in this study [14].

Different arabinoxylan hydrolysates exert differential effects on LPS-induced IL-8 and TNF-α release in colon cancer cell lines (Caco-2 and HT-29 cells) [39]. Feedstuffs containing arabinoxylans, mannose or galactomannans are able to decrease the E-coli-induced IL-1β, IL-8 and TNF-α expression in a porcine intestinal epithelial cell line (IPEC-J2) [37].

The effect of dietary fiber on intestinal DCs

Several *in vitro* studies have shown that dietary fiber promotes immunological responses by acting on intestinal DCs. For example, a GOS/FOS mixture stimulated IL-10 release by human monocyte-derived DCs [40], whereas pectin reduced their synthetic lipopeptide P3CSK4-induced release of IL-6 and IL-10 [41]. Different dietary fiber (GOS, inulin, pectin, arabinoxylan, β-glucan) caused a more regulatory status of DCs as indicated by an elevated IL-10/IL-12 ratio [42, 43]. The immunomodulating effects of dietary fiber on DCs might be dependent on its interaction with (released factors from) IECs as described by Bermudez-Brito *et al*. using co-culture systems. It has been shown that production of different cytokines and chemokines, including IFN-γ, IL-12, IL-1, IL-6, IL-8, MCP-1, MIP-1α, RANTES and TNF-α, released by DCs is decreased after incubation with dietary fiber-stimulated Caco-2 cell medium (GOS, inulin, pectin, arabinoxylan, β-glucan, resistant starches) [42, 44].

The effect of dietary fiber on intestinal macrophages

Dietary fiber has been shown to stimulate basal proinflammatory cytokine release by macrophages and monocytes. FOS and inulin reportedly induced TNF-α and IL-10 release by rat and human monocytes [45]. Stimulation of murine peritoneal macrophages with oat β-glucan resulted in the production of IL-1 [46], and an increase in phagocytic activity [47]. In RAW264.7 cells, a murine macrophage cell line, pectin decreased the P3CSK4-induced IL-6 production [41], and arabinoxylan hydrolysates modulated the LPS-induced NO-production [36].

The effect of dietary fiber on intestinal MCs

Dietary fiber can inhibit MC activation. Angelica polysaccharides (AP) inhibited the release of proinflammatory cytokines, such as IL-1, TNF-α, IL-6, IL-4, and

MCP-1 and also possessed inhibitory effects on allergic mediator release, including β-hexosaminidase and leukotrienes C4 and histamine, by RBL-2H3 MCs [48]. These effects might be related to an AP-induced downregulation of essential proteins in the Gab2/PI3K/Akt and Fyn/Syk pathways and/or the AP-induced decrease in Ca^{2+} influx [48]. Two different sulfated polysaccharides repressed the IgE-mediated activation in the same cell line [49]. The HMO, 6'SL, inhibited the degranulation of murine bone marrow-derived MCs, whereas 2'FL had no effect [50].

The effect of dietary fiber on blood mononuclear cells

In addition, dietary fiber may promote the development of the intestinal immune system as suggested by studies done on cord blood mononuclear cells from healthy newborns. Low-molecular-weight fucoidan (LMWF) stimulated IL-4 and IL-13 production in these cells, whereas the acidic oligosaccharides increased IL-13 production and the percentage of IFN-γ producing T cells. Both types of dietary fiber significantly increased CD25 expression, indicative of their effects on lymphocyte function and maturation [51]. Acidic oligosaccharides skewed the Th-2 type T-cell phenotype towards a more balanced Th-1/Th-2 profile in cord blood cells as well as in peripheral blood mononuclear cells (PBMCs) from adults [52]. In addition, shorter chain fructans induced a more regulatory cytokine balance compared to longer chain fructans as measured by IL-10/IL-12 ratios in human PBMCs [53].

Potential molecular mechanisms of the regulation of intestinal immunity by dietary fiber

Dietary fiber binds to carbohydrate-binding domains

The immune-modulatory effects of dietary fiber on macrophages and DCs are mediated, at least in part, by binding to carbohydrate receptors, including Ca^{+}-dependent C-type lectin receptors, such as mannose receptor (MR), DC-SIGN, macrophage galactose-specific lectin (MGL) and langerin, and Ca^{+}-independent C-type lectin receptors, like dectin-1, dectin-2 and complement receptor 3 (CR3). These receptors can recognize mannose, β-glucans, fucose-containing glycans, glucose, galactose and/or chitin [54]. After binding, intracellular signaling pathways are activated, ultimately leading to the modulation of immune responses [55, 56].

HMOs exhibit similarities to selectin ligands, and therefore, possibly bind to different selectins, known as carbohydrate-binding proteins, which support

leukocyte adhesion to the blood vessel wall [57]. HMOs are complex glycans, that interact with galectins [58]. Galectins also contain carbohydrate-recognition domains and serve as pattern recognition receptors (PRRs) for dietary fiber. For example, intestinal epithelium-derived galectin-9 was involved in the immunomodulating effects of nondigestible oligosaccharides as demonstrated in a co-culture model with IECs and peripheral blood mononuclear cells [59].

Dietary fiber binds to TLRs

Various types of dietary fiber act as TLR ligands, with downstream phosphorylation of IκB and effects on cytokine production**.** Two different types of resistant starches (RS), including RS type 2 and type 3, predominantly bind to TLR2, and TLR2 and TLR5, respectively [44]. ITF can bind to TLR2 located on IECs [26] and PBMCs, and to a lesser extent TLR4, 5, 7, 8, and nucleotide-binding oligomerization domain-containing proteins 2 (NOD2) on PBMCs, which resulted in NF-κB/AP-1 activation [53]. The chain length of ITF is important for TLR binding and the induced activation pattern, including cytokine release [53, 60]. Interestingly, pectin inhibited TLR2-TLR1 activation [41].

GOS, FOS and inulin are TLR4 ligands in IECs and induced cytokine production dependent on TLR4/MyD88/NF-κB and secondarily on MAPK [31]. Monocytes and DCs can also bind GOS, FOS and inulin via TLR4 [40, 45]. Different HMOs controlled immune responses via TLR3 and TLR4 signaling as reviewed by He *et al*. [61].

CD14 expression in enterocytes mediated LPS-TLR4 stimulation and corresponding IL-8 response and 2'FL suppressed CD14 expression and repressed NF-κB and p-ERK levels, whereas signal transducers and activators of transcription 3 (STAT3) phosphorylation and expression of IκB and suppressor of cytokine signaling 2 (SOCS2) were increased [35].

In summary, dietary fiber binds and activates different TLRs, mainly TLR2, 3 and 4, in a cell type-independent manner.

Activation of peroxisome proliferator-activated receptor γ by dietary fiber

Previous research has highlighted the role of peroxisome proliferator-activated receptor γ (PPARγ) in regulating inflammation and immunity [62]. Oligosaccharides (α3-sialyllactose, or FOS) exerted an anti-inflammatory effect by activation of PPARγ, which was at least partly dependent on one of the members of the novel family of pattern recognition molecules in innate immunity, the peptidoglycan recognition protein 3 (PGlyRP3) [32]. PGlyRPs is expressed in enterocytes and can recognize microbes. In contrast to TLRs and NODs,

PGlyRP3 seems to have an anti-inflammatory role in intestinal epithelial cells. This anti-inflammatory effect is mediated via NF-κB pathway suppression, as also observed in reduced gene expression and nuclear translocation of NF-κB induced by α3-sialyllactose and FOS [32, 63]. The anti-inflammatory effect of 6'SL was PPARγ-dependent and associated with a decreased activity of the transcription factors AP-1 and NF-κB [34].

Taken together, dietary fiber can alter intracellular signaling via binding to specific cell surface receptors on different cell types that regulate epithelial cell maturation, barrier function and mucosal immunity (summarized in **Table 1**). The mechanisms and scope of these effects are determined by the chemical structure, the purity and the origin of dietary fiber and its target cell type.

In vivo relevance

Although intestinal epithelial cell lines, including Caco-2 and HT-29, in general provide a powerful tool to investigate properties and molecular mechanism of the intestinal epithelium, results gained from these *in vitro* models can be difficult to extrapolate to the (human) *in vivo* situation. Despite the abundant literature concerning microbiota-independent effects is related to *in vitro* studies, an *in vivo* study with germ-free mice by Lleywellyn *et al*. showed that psyllium fiber has both microbiota-dependent and independent effects and reduced the severity of colitis related to an improved intestinal barrier function and stimulation of the innate and antimicrobial immunity, confirming the biological relevance of microbiota-independent effects of dietary fibers [64]. However, the effect on colitis of psyllium fiber was significantly greater in SPF animals. Other *in vivo* studies with dietary fibers using germ-free mice highlighted the importance of the microbiota to maintain gut homeostasis and to prevent diseases [65, 66].

Table 1. Microbiota-independent effects of dietary fiber on intestinal barrier function and immunity.

Dietary fiber	Cell type	Effect	Mechanism	Ref
Acidic and neutral human milk oligosaccharides	HT-29, HIEC, Caco-2 cells	Proliferation	Interaction with EGFR Modulation of downstream EGFR/Ras/Raf/ERK	[19]
Galacto-oligosaccharides	LS174T cells	Barrier	Stimulation of goblet cells	[15]
Chitosan oligosaccharides	T84 cells	Barrier Immune	AMPK activation via CaSR-PLC-IP3 receptor channel-mediated calcium release AMPK-independent inhibition of NF-κB signaling	[24]
Human milk oligosaccharides, galacto-oligosaccharides, inulin, inulin-type fructans, fructo-oligosaccharides, α3-sialyllactose	T84, NCM-460, H4, IEC18, Caco-2 cells, reporter cells	Immune Barrier	Modulation NF-κB signaling	[26, 31-34, 53, 60]
Resistant starches (α-glucan-based)	Human DCs, reporter cells, Caco-2 cells	Immune	TLR2 and TLR5 binding	[44]
Inulin-type fructans, lemon pectins	Reporter cells, T84 cells	Immune Barrier	Binding to TLR2 (and to a lesser extent to TLR4, 5, 7, 8, and NOD2)	[25, 26, 53]
Pectin	Human DCs, mouse macrophages	Immune	TLR2 binding Inhibition of TLR2–TLR1 pathway	[41]
Fructo-oligosaccharides, inulin, galacto-oligosaccharides	Rat monocytes, human monocyte derived DCs, IEC18, HT-29, Caco-2 cells	Immune	TLR4 binding	[31, 40, 45]
Human milk oligosaccharides, 2'fucosyllactose	T84 cells, H4 cells	Immune	Suppressing CD14 expression Modulation of downstream TLR4-LPS-CD14	[35]
Galacto-oligosaccharides, (chicory) inulin, inulin-type fructans, sugar beet pectin, wheat arabinoxylan, barley β-glucan	Human DCs, Caco-2 cells, IEC18 cells, reporter cells	Immune	MyD88 dependent	[31, 42, 53]
Human milk oligosaccharides, α3-sialyllactose, fructo-oligosaccharides	T84 cells, Caco-2 cells	Immune	PPARγ dependent	[32, 34]
Mannose, β-glucans, fucose-containing glycans, glucose, galactose, chitin	DCs, macrophages	Immune	Binding to C-type lectin receptors	Reviewed in [54]
Galacto-oligosaccharides, fructo-oligosaccharides	HT-29 and T84 cells, human PBMCs	Immune	Galectin-9	[59]
Angelica polysaccharides	Mast cells	Immune	Modulation of Gab2/PI3-K/Akt and Fyn/Syk pathways	[48]

Microbiota-dependent effects of dietary fiber on gut homeostasis

The human gut microbiome is constituted by numerous microbial communities comprising hundreds of individual bacterial species, including beneficial (non-invasive) microbes, (e.g., *Bifidobacterium* and *Lactobacillus* spp.) and potential pathogenic (invasive) microbes (e.g., *Escherichia coli*) [67]. *Bifidobacterium* and *Lactobacillus* spp. have anti-inflammatory effects [68], stimulate brain functions [69], modulate metabolism [70], and antagonize intestinal pathogens [71]. Pathogenic microbes, such as enteropathogenic and enterohemorrhagic *E. coli* and *C. difficile*, can cause gut pathology by producing toxins and causing infections. Dietary fiber can help to maintain intestinal homeostasis and to prevent diseases by promoting and limiting the growth and effects of beneficial and pathogenic microbes, respectively (summarized in **Table 2**). The majority of data supporting the microbiota-dependent effects of dietary fiber are based on *in vitro* and *in vivo* animal studies, while well-designed clinical trials are scarce (**Table 2**).

Table 2. Microbiota-dependent effect of dietary fiber on gut homeostasis.

Dietary Fiber	Type of study	Microbiome changes	Mechanisms	Diseases	Ref
Arabinoxylan-oligosaccharides	*In vivo; Clinical study*	*Bifidobacterium* ↑ *Lactobacillus* ↑	*Bifidobacterium* and/or *Lactobacillus* stimulation: (1) improving intestinal barrier function, (2) immunomodulation, (3) influencing brain function through the production of neuropeptides, (4) antimicrobial activities.	Metabolic syndrome; Metabolic endotoxemia; IBD; Diabetes; Obesity; Food poisoning; Irritable bowel syndrome; Anxiety	[72-83]
Galacto-oligosaccharides	*In vivo; In vitro; Clinical study*				
Fructo-oligosaccharides	*In vivo; In vitro; Clinical study*				
Inulin-type fructans	*In vivo*				
Human milk oligosaccharides	*In vivo; Clinical study*				
Laminarin	*In vivo*				
Resistant starches	*In vivo*				
Galacto-oligosaccharides	*In vitro*	*Vibrio cholerae* ↓ *Entamoeba histolytica* ↓	Inhibition of pathogen-induced cytotoxicity: (1) direct binding to toxins or lectins, (2) inhibiting the expression of toxins or toxin-related genes, (3) neutralizing or interfering with toxins.	Diarrhea; GI disease; *E. histolytica* infection; Urinary tract infections; Childhood shigellosis; Colitis	[84-91]
Human milk oligosaccharides	*In vitro*	Uropathogenic *Escherichia coli* ↓ *Clostridium difficile* ↓ *Candida albicans* ↓			
Fructo-oligosaccharides	*In vivo*	*Clostridium difficile* ↓			
Inulin-type fructans	*In vivo*	*Clostridium difficile* ↓			
Pectic-oligosaccharides	*In vitro*	*Escherichia coli* ↓			
Pectin	*In vitro*	*Escherichia coli* ↓			
Resistant starches	*Clinical study*	*Shigella* ↓			
Galacto-oligosaccharides	*In vivo; In vitro*	*Escherichia coli* ↓ *Entamoeba histolytica* ↓ *Cronobacter sakazakii* ↓ *Citrobacter rodentium* ↓	Anti-adhesion: (1) anti-adhesive, (2) decoy glycan effects.	Enteric infection; GI disease; Diarrhea; *E. histolytica* infection; Food poisoning	[84, 87, 88, 92-95]
Human milk oligosaccharides	*In vitro*	*Entamoeba histolytica* ↓ *Campylobacter jejuni* ↓			
Pectic-oligosaccharides	*In vitro*	*Escherichia coli* ↓			
Pectin	*In vitro*	*Escherichia coli* ↓			
Human milk oligosaccharides	*In vitro*	*Streptococcus agalactiae*↓	Growth inhibition: (1) glycosyltransferase catalysis, (2) antimicrobial activities, (3) pore formation and permeabilization of cell walls, (4) blockage of nutrient flow.	Invasive bacterial infections in newborns; GI disease	[96-98]
Pectic-oligosaccharides	*In vitro*	*Clostridium perfringens*↓ *Bacteroides fragilis*↓			
Chitosan oligosaccharides	*In vitro*	*Bacillus cereus* ↓ *Escherichia coli* ↓			

Dietary fiber increases Bifidobacterium and/or Lactobacillus spp. growth

Dietary fiber intervention, especially with ITF, FOS and GOS, have been widely used to stimulate the growth of *Bifidobacterium* and/or *Lactobacillus* species and the production of SCFAs [66]. There are different mechanisms of action related to the beneficial effects of dietary fiber-stimulated *Bifidobacterium* and *Lactobacillus* spp. growth on human health: (1) improving intestinal barrier function, (2) immunomodulation, (3) influencing brain function, and (4) antimicrobial activities.

Bifidobacterium and *Lactobacillus* spp. have been shown to improve intestinal epithelial barrier function [99]. An increase in the number of *Lactobacillus* and *Bifidobacterium* spp. in the cecum induced by oligofructose supplementation controls endogenous production of the intestinotrophic hormone, glucagon-like peptide-2 (GLP-2), and consequently improved gut barrier function in obese mice [73].

Studies in healthy humans indicated that wheat bran extract containing arabinoxylan oligosaccharides (AXOS) can increase fecal *Bifidobacteria* levels and exert beneficial effects on gut health parameters [74]. The growth of *Bifidobacteria* induced by fructans in the diet was associated with an increase in villus height and crypt depth, resulting in thicker mucosal layers in the rat jejunum and colon [100].

Gut commensal microbes can also affect the host immune system. *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* increased the number of regulatory T cells (Tregs) and inhibited the development of dermatitis and asthma, respectively [101, 102]. Food-derived laminarin (a kind of glucan-polysaccharides), an antagonist of the Dectin-1 receptor (a C-type lectin receptor expressed on DCs and macrophages), inhibited the development of DSS-colitis via promoting the growth and colonization of *Lactobacillus murinus* leading to an increase in Tregs [75]. In addition, the administration of inulin and FOS decreased intestinal inflammation and reduced the severity of colitis, which was associated with increased butyrate, lactate and *lactobacilli* spp. in the colon [76, 77].

The oral administration of *Lactobacillus rhamnosus* and *Bifidobacterium lactis* enhanced NK cell-mediated tumoricidal activity in mouse spleens [103] and improved the ability of the elderly to resist immune aging [68]. Gopalakrishnan *et al.* reported that GOS can reduce the severity of colitis manifested by less infiltration of inflammatory cells in the cecum and colon and a reduction in goblet cell depletion, which was mainly because of a GOS-induced increase in *Bifidobacterium* spp. leading to enhanced NK cell function and IL-15 production [78].

Bifidobacterium strains exhibited anti-inflammatory and immuno-regulatory effects and increased IL-10 release by DCs and decreased IFN-γ production by T cells [104]. A clinical study in patients with Crohn's disease showed that FOS stimulated the growth of *Bifidobacteria* in both feces and mucosa, and these changes were related to immunoregulatory effects on mucosal DC functions and IL-10 release [79].

In a large prospective study, it has been observed that long-term intake of dietary fiber is associated with a lower risk of Crohn's disease [105]. Arslanoglu *et al*. suggested that early nutritional intervention with GOS/FOS seems to modulate the infant's immune system, leading to a protective effect against allergy and infection [106]. In addition, 2'FL stimulated immune development in infants as observed by lower plasma cytokine profiles, such as IL-1, IL-6 and TNF-α [83].

Furthermore, intestinal bacteria, including *Lactobacillus* and *Bifidobacterium* spp., importantly modulate brain functions and neuropsychiatric diseases [69]. Dietary fiber can modulate brain signaling, via the microbial gut-brain axis, control emotional behavior and ameliorate cognitive and behavioral disorders in animal models and humans, in part, by increasing *Lactobacillus* and *Bifidobacterium* spp. gut populations [107, 108]. The administration of a probiotic formulation consisting of *L. helveticus* and *B. longum* exhibited anxiolytic-like activity in rats and beneficial remission of psychological distress in healthy women [109]. SCFAs, primarily acetate, have been shown to cross the blood-brain barrier and affect brain function. Acetate derived from the fermentable carbohydrate inulin directly suppressed appetite by activating the enzyme acetyl-CoA carboxylase in the brain and by producing regulatory neuropeptides [80]. In patients with irritable bowel syndrome (IBS), GOS stimulated gut *Bifidobacteria* and alleviated bloating symptoms, and reduced anxiety scores [81]. HMOs (3′SL or 6′SL) that promote bifidobacterial growth and modulate inflammatory responses, prevented stress-induced changes in gut microbiota, and diminished anxiety-like behavior, potentially via modulating the microbial gut-brain axis [82].

Lactobacilli and *Bifidobacteria* exhibit adhesion properties that interfere with the adhesion of bacterial pathogens to IECs and participate in the antimicrobial activity of host GI defense [71]. Two different *Bifidobacterium* strains isolated from infant feces inhibited the entry of *S. enterica* serotype *Salmonella typhimurium* into Caco-2 cells [110]. Different *Lactobacillus* spp. (e.g., *Lactobacillus plantarum)* exhibited an anti-*Salmonella* effect via antimicrobial activity and competitive adhesion to mucin and HT-29 cell lines. Brink *et al*. showed that the prebiotic saba starch functioned better than *Lactobacillus plantarum* against *Salmonella* in

vitro [111, 112]. Moreover, *Lactobacillus* spp. cultured and fermented with FOS showed antimicrobial activity against *Listeria innocua* and *Enterobacter aerogenes* [72].

There is abundant literature concerning the effects of dietary fiber on the growth of *Bifidobacterium* and *Lactobacillus* spp. [113], however dietary fiber can also stimulate other microbes. For example, COS inhibited gut dysbiosis by promoting *Akkermansia muciniphila* [114], whereas inulin has been found to increase faecal *Faecalibacterium prausnitzii* in healthy humans [115].

In summary, dietary fiber regulates the abundance of important beneficial commensal bacteria in the GI tract, such as *Bifidobacterium* and *Lactobacillus* spp., thereby improving intestinal barrier function, immune regulation, antimicrobial activity and neuropsychiatric function.

Dietary fiber inhibits pathogen-induced cytotoxicity

Dietary fiber can also interfere with pathogenic microbes, for example, toxin-producing microbes. Dietary fiber protects from the effects of pathogenic microbes by (1) directly binding to toxins or lectins, (2) inhibiting the expression of toxins or toxin-related genes, and (3) neutralizing or interfering with toxins.

C. difficile toxin A and toxin B have carbohydrate binding sites that bind to HMOs [116]. This suggests that HMOs can specifically abolish cytotoxicity by modifying the diethylpyrocarbonate of histidine residues in toxins [85]. Furthermore, HMOs also have the ability to bind to Shiga toxin type 2 holotoxin (Stx2) and the B subunit homopentamers of cholera toxin, heat-labile toxin and Shiga toxin type 1 (CTB5, HLTB5 and Stx1B5) [116]. GOS are usually added to infant formula to simulate the beneficial effects of HMOs. GOS and HMOs both contain terminal galactose (Gal), which can bind to specific lectin of protozoan parasite *Entamoeba histolytica*. HMOs and/or GOS rescued the destruction of IECs by binding to the galactose/N-acetylgalactosamine (Gal/GalNAc) lectin on the surface of *E. histolytica* trophozoites to reduce the cytotoxicity and adhesion of *E. histolytica* to intestine [87].

HMOs decreased invasion and toxicity of *Candida albicans,* a fungal colonizer of the neonatal gut, by shortening hyphal length and by reducing the expression of hyphal-specific genes (mycelial cell wall protein-encoding genes), possibly protecting premature infants from *C. albicans*-induced intestinal disorders [86]. ITF and FOS decreased gene expression of *Clostridium* cluster XI and *Clostridium difficile* toxin B (TcdB) in the feces of rats in an IBD model, thereby reducing chronic inflammation [89].

Pectin and pectic-oligosaccharides (POS) were shown to neutralize and interfere with toxins and reduce cytotoxicity of Shiga-like toxins from *Escherichia coli* O157:H7 *in vitro*, as well as protect cells from Shiga-like toxin-induced damage [88, 90]. A clinical study in patients with severe bloody dysentery caused by *Shigella* infection showed that green banana (rich in RS) improved childhood shigellosis, reduced myeloperoxidase activity, mucus, and blood, and increased SCFA concentrations in feces [91].

Dietary fiber acts as anti-adhesives and decoy glycan receptors

Soluble decoy carbohydrates can bind to pathogenic microbes, thereby preventing their binding and damage to the gut epithelium. The carbohydrate moiety on epithelial cells serves as a binding site for adhesins expressed by many intestinal pathogens such as *Salmonella*, enteropathogenic *Escherichia coli* and enterohemorrhagic *Escherichia coli*. GOS act as anti-adhesives that competitively inhibit the adhesion of pathogens to GI epithelium. *In vitro* studies showed that the adherence of enteropathogenic *Escherichia coli* and *Cronobacter sakazakii* to host epithelial cells can be inhibited by GOS [92, 93]. In addition, GOS competitively inhibited *vibrio cholerae* toxin binding to its cell-surface toxin receptor (GM-1) on the host cell surface [84]. In addition, GOS significantly reduced the adhesion of the murine bacterial pathogen *Citrobacter rodentium* to the epithelial cell surface in a dose-dependent manner *in vitro*, but not *in vivo*, which might be related to the expression of adhesins that are insensitive to GOS when *C. rodentium* grows *in vivo* [94].

Similar to GOS, HMOs also act as anti-adhesives and decoy glycan receptors, preventing adherence of pathogens to host intestinal epithelium, thereby decreasing the risk of infections [87, 117]. Breastfeeding results in a lower incidence of diarrhea, which could be related to the reduced binding and colonization of the intestinal pathogen *Campylobacter jejuni* induced by HMOs [117]. In addition, the anti-adhesive activity of HMOs (as well as GOS) also prevented the attachment, invasion and infection of *Entamoeba histolytica* to human IECs [87]. HMOs and GOS bind to Gal/GalNAc lectin on the surface of *Entamoeba histolytica* to interfere with its specific binding site, and this anti-adhesive and cytoprotective effect depends on the free terminal Gal.

Di *et al.* reported that pectin and POS blocked the binding of *E. coli* O157:H7 to human HT-29 cells. POS have the ability to compete with Shiga toxin 2 binding to the neutral glycolipid globotriaosylceramide Gb3 receptor on the human IEC surface. Low molecular weight and deesterification of POS facilitates the anti-

adhesive activity of Shiga toxin-producing *Escherichia coli* to human IECs [88]. Moreover, POS concentration-dependently inhibited the adherence and invasion of *Campylobacter jejuni* to Caco-2 cells [95].

Dietary fiber inhibits the growth of pathogenic microbes

Notably, some types of dietary fiber exhibit anti-bacterial effects and directly inhibit the growth of pathogenic microbes independent of host immunity. For example, non-sialylated HMOs directly inhibited the growth of *Streptococcus agalactiae*. This inhibition was specific for *Streptococcus agalactiae* and not observed for uropathogenic *Escherichia coli*, *Pseudomonas aeruginosa*, or *Staphylococcus aureus* [96]. Moreover, this growth inhibition by HMOs was dependent on the expression of bacterial glycosyltransferase encoded by the gbs0738 gene in *Streptococcus agalactiae.* This glycosyltransferase might catalyze the incorporation of HMOs components into the cell wall of bacteria to inhibit the growth of *Streptococcus agalactiae* [96]. POS completely inhibited the growth of *C. perfringens* and *B. fragilis* strains, whereas FOS enhanced the growth of *C. perfringens* and *B. fragilis* strains. This indicates that POS and HMOs not only display prebiotic properties, but also exhibit antimicrobial activities [97]. Another study found that COS showed better growth inhibitory activity against both Gram-positive and -negative bacteria, such as *B. cereus, E. coli, Y. enterocolitica* and *B. licheniformis,* compared to natural chitosan. Higher DP COS (monomers) with a low degree of acetylation exhibited optimal growth inhibition and lead to pore formation and permeabilization of *Bacillus cereus* cell walls. In addition, the blockage of nutrient flow caused by aggregation of COS was the main cause of growth inhibition and lysis of *E. coli* [98].

Although only a few *in vitro* studies have shown that dietary fiber directly inhibits the growth of pathogenic microbes, it may open new therapeutic avenues for the treatment of pathogen infections and may contribute to reducing the use of antibiotics.

Dietary fiber-derived microbial metabolite production

A substantial part of the potential health benefits of dietary fiber is related to its metabolites produced by the gut microbiota. The most prominent dietary fiber-derived metabolites are SCFAs, including acetate, propionate, and butyrate, which are found in high concentrations in the intestine, reaching around 13 mmol/kg intestinal content in the terminal ileum, increasing to 131 mmol/kg in the caecum, with lower concentrations in the distal colon (80 mmol/kg) [118]. The amount and types of SCFAs that are produced largely depend on substrate availability, bacterial species composition of the microbiome and intestinal transit time [119]. Acetate, propionate, and butyrate are the most abundant SCFAs (≥95%) in the GI tract [120]. Generally, Bacteroidetes are potent producers of acetate and propionate, whereas butyrate is mainly produced by Firmicutes [119, 121].

Potent substrates for SCFAs production are polysaccharides, oligosaccharides, proteins, peptides and glycoprotein precursors by anaerobic micro-organisms [122], although in quantitative terms the carbohydrates (polysaccharides, oligosaccharides) are most important for SCFAs production.

The effect of SCFAs on gut homeostasis

Similar to dietary fiber, SCFAs may contribute to gut homeostasis by regulating mucus (over)production and secretion [123]. Indeed, *in vitro* studies indicated butyrate and propionate as inducers of mucin (MUC2) synthesis and secretion by intestinal cells [124]. Furthermore, SCFAs preserved intestinal barrier function by decreasing tight junction permeability [125], potentially through activation of AMPK [126]. Although the authors compared the effects of butyrate to similar effects of (low concentrations of) histone deacetylase (HDAC) inhibitor Trichostatin A (TSA), a recent *in vivo* study suggested butyrate to promote tight junction protein expression in a G protein-coupled receptor (GPR)109A-dependent manner [127]. Finally, SCFAs promoted gut health by decreasing local pH, thereby inhibiting or promoting the colonization of different taxa [128].

Regulation of gut immunity and systemic pathology by SCFAs

SCFAs regulate local immunity and systemic pathology. Interestingly, these microbial metabolites are connected to (novel) molecular pathways [6].

SCFAs as HDAC inhibitors

Butyrate, and to a lesser extent propionate, is a potent HDAC inhibitor in intestinal cells and associated with the regulation of intestinal immunity and gut homeostasis [129]. Via HDAC inhibition, butyrate and other SCFAs display anticancer activities by inducing apoptosis and differentiation [130]. Interestingly, cancerous colonocytes shifted their primary energy source from butyrate to glucose, resulting in a 3-fold accumulation of butyrate within the cell (known as the Warburg effect) inhibiting the proliferation of the cancerous colonocytes [131]. Butyrate modulated the function of intestinal macrophages via HDAC-mediated downregulation of proinflammatory mediators, including nitric oxide, IL-6, and IL-12 [132]. In 2013, two reports suggested that intestinal immunity is promoted by butyrate via histone H3 acetylation, as well as via the generation of colonic Tregs in a GPR43-dependent manner [133, 134]. Remarkably, the co-occurring HDAC inhibition and stimulation of GPR receptors by SCFAs both diminished endothelial cell activation, in a similar fashion [135]. More distant from the gut, elevated acetate levels were shown to reduce allergic airway disease in mice by enhancing Tregs in the lung through HDAC9 inhibition [136]. Taken together, butyrate and other SCFAs can modulate immune cell function and disease, holding potential clinical implications.

SCFAs as G protein-coupled receptor ligands

SCFAs are ligands for cell membrane receptors such as GPR43 (also called FFA2), GPR41 (also called FFA3) and GPR109a (also called hydroxycarboxylic acid receptor 2 (HCA2)). GPR43 can potently be stimulated by acetate, propionate and butyrate, while GPR41 is a more potent receptor for butyrate and propionate [137]. Two mice *in vivo* studies showed that butyrate suppresses colonic inflammation and carcinogenesis via GPR109a [138]. Singh *et al*. showed that butyrate-stimulated signaling of GPR109a increases the generation of mouse Tregs, elevates IL-18 secretion by IECs, and increases IL-10 production by T cells [138]. An interesting study by Maslowski *et al*. showed that administration of acetate to the drinking water of mice could diminish inflammatory responses in models of colitis, arthritis and allergic airway disease in a GPR43-dependent manner [139]. Similarly, administration of propionate reduced allergic airway disease in mice via hematopoiesis of DCs and reduced Th2 effector function in a GPR41-dependent manner [140]. Due to the ubiquitous expression of the receptors on different cell types, and the reported anti-cancer and -inflammatory effects of SCFA-mediated GPR stimulation, the pharmacological development of GPR-selective compounds might be of therapeutic interest [6].

SCFAs as regulators of transcriptional activity

Nuclear PPARs can be stimulated by SCFAs with a potency order of butyrate > propionate > acetate [141]. Byndloss *et al*. showed that PPARγ inhibit dysbiotic Enterobacteriaceae expansion when stimulated by butyrate [142]. Butyrate can also regulate transcription factor activity directly: NF-κB activity is strongly inhibited by butyrate demonstrating therapeutic potential in experimental models of colitis and Crohn's disease [143]. Furthermore, butyrate-regulated genes often contain several binding sites for specificity protein 1 (SP1) and AP-1, suggesting that butyrate (and to a lesser extent propionate) can exert its effects via activation of these transcription factors and associated pathways [144, 145]. Finally, the aryl hydrocarbon receptor (AhR) emerged as a critical regulator of immune and metabolic processes in the GI tract. Recently, butyrate was found to induce AhR activity and transcription of AhR- dependent genes in human IECs [146].

Taken together, dietary fiber-derived SCFAs are important promotors of colonic health and positive regulators of immune responses.

Concluding remarks and future perspectives

This review describes the impact of different dietary fiber on the microbiome composition, but also emphasizes the microbiota-independent effects (**Figure 2**). It has been well demonstrated that dietary fiber promotes commensal bacteria in the GI tract, such as *Bifidobacteria and Lactobacilli*. *Bifidobacteria and Lactobacilli* have been shown to directly compete with pathogenic bacteria, but can also stimulate the intestinal barrier, immune and brain function. These dietary fiber do not only stimulate commensal bacteria, but also directly influence pathogens via inhibition of pathogen growth, competitive inhibition of adhesion, abolishment of cytotoxicity and toxin production induced by pathogens, which have been associated with a range of beneficial health effects. A substantial part of these potential health benefits is related to dietary fiber-derived microbial metabolite production, including SCFAs. SCFAs can promote intestinal immune homeostasis through inhibition of HDAC and via several other receptor-mediated pathways, such as GPR41, GPR43, GPR109a and PPARs.

In addition, several microbiota-independent effects of dietary fiber are important for maintaining intestinal epithelial barrier function and promoting gut immune responses (**Figure 2**). Various dietary fiber can bind to receptors on epithelial cells (EGFR, PPARγ and TLRs) and different immune cells (TLRs, carbohydrate binding domains) or interact with regulatory molecules (e.g., AMPK) to regulate intestinal barrier function and immune homeostasis. These *in vitro* studies suggest that multiple dietary fiber exhibit diverse effects on intestinal epithelial cell-derived cytokine and chemokine production and release. This may be associated with the structure-function relationship among dietary fiber structure and their immunomodulatory properties. However, the different types of IECs and triggers used in the *in vitro* studies can contribute to heterogenicity between the studies. In addition, more *in vivo* studies with germ-free mice are needed to confirm the biological relevance of the microbiota-independent effects. The scope and mechanisms of its effects on gut homeostasis emphasize that dietary fiber does more than modifying the gut microbiota and highlight its multi-faceted potential in the treatment or prevention of (chronic) diseases.

The use of dietary fiber to maintain health and prevent diseases is rapidly expanding, and dietary fiber-based nutraceuticals and functional foods are explored for their effects on several diseases [147]. Both pharmacologists and nutritionists are increasingly aware of the importance of maintaining and restoring (gut) homeostasis and agree that multifactorial diseases require multi-targeting approaches combining pharmacology and nutrition [148]. The new developments in nutraceutical and functional food research using omics techniques have gained

interest in the concept of personalized nutrition [149], which seems to be promising for dietary fiber as well. Especially, because the significant interindividual variability in response to dietary fiber intake, which likely depend on microbiome variability [150]. Since most of the studies performed so far focus on the effects of individual dietary fiber, it will be a major challenge to study the interaction of different types of dietary fiber and the interaction of dietary fiber with other nutritional components.

In this respect, well-conducted *in vitro*, *in vivo* and clinical studies are needed to establish the efficacy and mechanisms of action of dietary fiber in treating patients and in utilizing dietary fiber-based approaches for the prevention of disease.

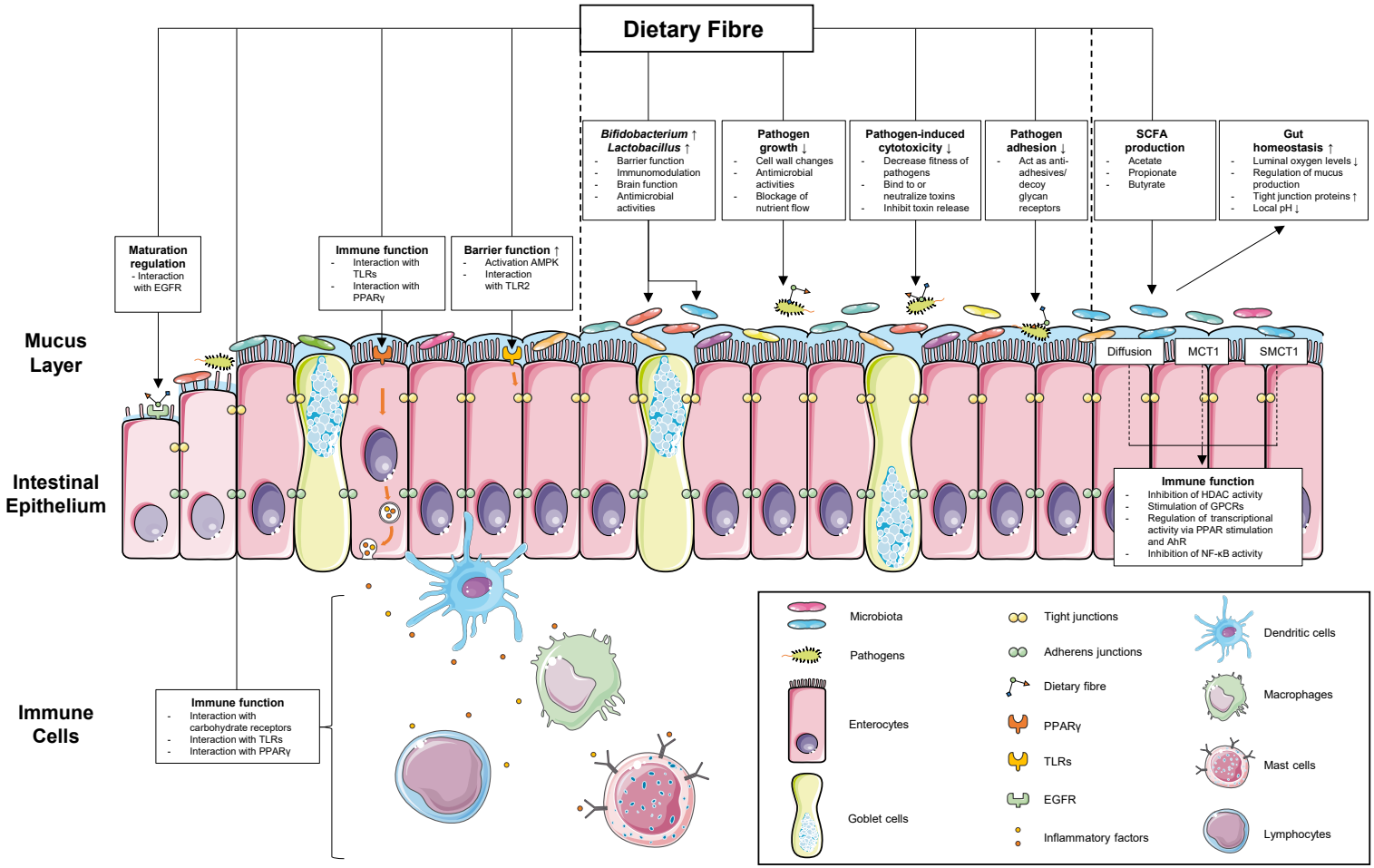

Figure 2 Mechanisms of microbiota-dependent and -independent effects of dietary fiber. Dietary fiber promotes gut homeostasis by directly affecting the intestinal commensal microbiota composition (e.g., increased growth of *Bifidobacterium* and *Lactobacillus*), leading to increased barrier function, immunomodulatory effects, stimulating brain function and antimicrobial activities. Dietary fiber also

protects gut homeostasis by inhibiting the cytotoxicity, adhesion or growth of pathogenic microorganisms. Dietary fiber is fermented into SCFAs by the microbiota, and these SCFAs further maintain intestinal (immune) homeostasis by inhibiting HDAC, stimulating GPCRs or regulating the transcriptional activity. Importantly, dietary fiber exhibits a microbiota-independent effect on the intestinal epithelium and different immune cells, including, dendritic cells, macrophages, mast cells and lymphocytes. These microbiota-independent effects include regulating epithelial cell maturation, enhancing epithelial barrier function and stimulating the intestinal immune system via specific PPRs on epithelial cells (EGFR, PPARγ and TLRs) and immune cells (TLRs, carbohydrate receptors) or via interaction with regulatory molecules (e.g., AMPK). *AMPK, AMP-activated protein kinase; AhR, aryl hydrocarbon receptor; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; HDAC, Histone deacetylase; MCT1, monocarboxylate transporter 1; NF-κB, nuclear factor kappa B; PPAR, peroxisome proliferator-activated receptor; SCFAs, short-chain fatty acids; SMCT1, sodium-coupled monocarboxylate transporter 1; TLRs, Toll-like receptors.*

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY [151], and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 [152].

Acknowledgements/funding statement

This work was partly funded by the public-private partnership 'CarboKinetics' coordinated by the Carbohydrate Competence Center (CCC, www.cccresearch.nl). CarboKinetics is financed by participating industrial partners Agrifirm Innovation Center B.V., Cooperatie AVEBE U.A., DSM Food Specialties B.V., FrieslandCampina Nederland B.V., Nutrition Sciences N.V., VanDrie Holding N.V. and Sensus B.V., and allowances of The Netherlands Organisation for Scientific Research (NWO).

Research grant funding was received from the China Scholarship Council for Y. Cai.

Conflicts of interest relevant: The authors declare no conflict of interest.

Abbreviations

2'FL, 2'fucosyllactose; 6'SL, 6'sialyllactose; AMPK, 5'AMP-activated protein kinase; AP, angelica polysaccharides; AXOS, arabinoxylan oligosaccharides; AP-1, activator protein-1; AhR, aryl hydrocarbon receptor; COS, chitosan oligosaccharides; CaSR, calcium-sensing receptor; CR3, complement receptor 3; DCs, dendritic cells; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides; GRO-α, growth-related oncogene-alpha; GLP-2, glucagon-like peptide-2; Gal/GalNAc, galactose/N-acetylgalactosamine; GI, gastrointestinal; HDAC**,** histone deacetylase; HCA2, hydroxycarboxylic acid receptor 2; HMOs, human milk oligosaccharides; IECs, intestinal epithelial cells; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; ITF, inulin-type fructans; LNnT, lacto-Nneotetraose; LMWF, low-molecular-weight fucoidan; MCs, mast cells; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein; MR, mannose receptor; MGL, macrophage galactose specific lectin; MCT1, monocarboxylate transporter 1; NODs, nucleotide-binding oligomerization domain-containing proteins; PRRs, pattern recognition receptors; PLC, phospholipase C; PBMCs, peripheral blood mononuclear cells; PGlyRP3, peptidoglycan recognition protein 3; POS, pectic-oligosaccharides; RS, resistant starch; SCFAs**, s**hort-chain fatty acids; STAT3, signal transducers and activators of transcription 3; SOCS2, suppressor of cytokine signaling 2; SMCT1, sodium-coupled monocarboxylate transporter 1; SP1, specificity protein 1; TSA, trichostatin A; TLRs, Toll-like receptors; Tregs, regulatory T cells; TEER, transepithelial electrical resistance; ZO-1, zonula occludens-1.

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9

Chapter 9

General Discussion and Summary

General Discussion

Respiratory infections

Respiratory infections, especially lung infections, are still one of the biggest causes of death in humans (children). In livestock species, particularly in calves, respiratory infections are one of the major causes of mortality [1-3]. In the presence of cofactors, such as adverse climatic conditions and air pollution, pathogens, such as human pathogen *Mycoplasma pneumoniae* and bovine pathogen *Mannheimia haemolytica*, grow explosively and invade into the lower respiratory tract of susceptible hosts, causing lung infections [4, 5]. This poses a great burden on both medical administrations and the costs associated with treatments. Treatment of bacterial pneumonia/infections is hampered by the increasing antimicrobial resistance worldwide [6], which requires new prophylactic and therapeutic strategies.

The respiratory infection model in calves has been used in research of human respiratory infections for decades [4, 7, 8]. As an animal model, the calf offers advantages in understanding the pathogenesis and effective interventions of lung infections: (I) calves and humans share similar clinical manifestations (e.g., cough reflex, lethargy) and anatomical characteristics (e.g., bronchial glands, cartilage airways), (II) calves and humans have similar pathological and immunological features (e.g., the influx of neutrophils into the lungs, the release of cytokines/chemokines by airway epithelial cells), (III) their large size facilitates repetitive sampling (e.g., blood and lung samplings) and standard-dose testing (e.g., the approximate dose for humans) and, (IV) most importantly, the prevalence of pneumonia in calves is extremely high [4]. Therefore, an *ex vivo* model based on the *M. haemolytica*/LPS-treated primary bronchial epithelial cells (PBECs) from calves and an *in vivo* model based on the naturally occurring respiratory infections in calves were described in **Chapter 2** and **Chapter 3**, respectively.

Lung epithelial cells and proinflammatory responses in respiratory infections

The respiratory epithelium acts as the first line of defense against inhaled pathogens through the physical barrier and its immunological functions [9]. Among them, epithelial cells are the central component of this physical barrier [10]. They are connected to each other by cell-cell junctions, including tight junctions and adherens junctions, forming an impermeable mechanical barrier to prevent invasion of pathogens [9, 10]. In **Chapter 2**, a well-developed physical

barrier has been observed in the primary culture of PBECs, manifested by a stable transepithelial electrical resistance (TEER) and high expression of tight junction protein ZO-1 and adherens junction protein E-cadherin.

In addition to physical protection, lung epithelial cells possess important immune functions in the defense of pathogens. For instance, lung epithelial cells rapidly recognize pathogens through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [10]. Upon PRR activation, lung epithelial cells not only generate cytokines/chemokines that recruit and activate leukocytes, but also produce endogenous danger signal molecules (e.g., ROS, ATP) [10, 11]. The activation of PRRs, the production of cytokines/chemokines and danger signal molecules induced by NDOs have been described in this thesis. In **Chapter 2**, the activation of TLR4/NF-κB pathway and subsequent release of cytokines/chemokines (e.g., IL-8, TNF-α, IL-6) by *M. haemolytica* and LPS in PBECs have been described. In **Chapter 3**, the production of cytokines/chemokines (e.g., IL-8, TNF-α, IL-6, IL-1β) in both blood and bronchoalveolar lavage fluid (BALF) and the influx of neutrophils from blood into the lungs have been observed in calves with lung infections. In **Chapter 4**, pathogens (e.g., *M. haemolytica*) induced the activation of NLRP3 inflammasome and caspase-1 via TLR4/NF-κB pathway and ROS/ATP production in PBECs and naturally exposed calves, stimulating the production of mature IL-1β.

Although the host has carefully designed the respiratory defense system, including a protective physical barrier, epithelial cell-mediated proinflammatory response, resident macrophages and recruited neutrophils, these defenses are susceptible to failure as a result of the presence of cofactors (e.g., stress, air pollution) and strategies adopted by pathogens (e.g., released virulence factors) [4, 5, 9]. In **Chapter 2**, the epithelial cells and barrier functions were damaged by *M. haemolytica*, with the consequence of pore formation on cell membranes and decreased epithelial integrity as confirmed by air-dried cytospins and TEER measurement. In **Chapter 3**, a numerous influx of neutrophils from the blood into the lungs was observed in the infected calves, accompanied by the depletion of macrophages and the continuous production of cytokines/chemokines. However, excessive neutrophils and proinflammatory cytokines result in inflammatory infiltration and injury in the lungs, leading to organ dysfunction and death, which was described in **Chapter 3**. Up to 80% of the calves showed lung lesions within 8 weeks after arriving at the facility.

Due to the failure of respiratory defenses, the strategies of pathogens, and the alarms of rising antimicrobial resistance, the development of and intervention with new alternatives is particularly important. The described pathogenesis of

lung infections in **Chapter 2** and **Chapter 3** suggests that potential interventions on impaired epithelial barriers and excessive proinflammatory responses might be very interesting.

Intervention strategies to suppress respiratory infections

1. Galacto-oligosaccharides (GOS)

Although the mechanism is not well described, GOS exhibit many beneficial effects on human health/diseases, including respiratory diseases [12, 13]. For instance, dietary GOS reduced pulmonary eosinophilic inflammation in a murine asthma model [13]. In **Chapter 4**, although no effects on clinical scores and symptoms were observed, consuming GOS relieved infection-induced lung inflammation in calves, including restored macrophage/neutrophil levels in BALF and decreased leukocyte levels in blood at an early stage. In addition, *M. haemolytica* positivity and local/systemic inflammation in calves with lung infections were also inhibited by oral GOS. The gut-lung axis, the interplay between the microbiota and the immune system, is thought to be involved in the GOS-related effects [14]. Though the microbiota was not investigated in the present study, GOS is well-known to stimulate the growth of intestinal commensal bacteria, e.g., *Bifidobacteria* and *Lactobacilli,* which can exert immunomodulatory and anti-inflammatory effects [12, 15].

Increasing evidence shows that GOS have the capacity to be absorbed into the systemic circulation (possibly in small amounts) after oral administration [16, 17], indicating that GOS might reach the lungs (bronchus) through blood circulation to exert direct effects on pathogens or host cells (e.g., airway epithelial cells). These microbiota-independent, direct effects of GOS were studied in a series of *in vitro* and *ex vivo* studies using PBECs and human lung epithelial cells, including 1) interference with TLR4-mediated signaling, 2) anti-oxidative effect, 3) neutralization or interference with bacterial toxins, 4) anti-bacterial effect, and 5) anti-adhesion or anti-invasive properties.

1.1 Interference with TLR4-mediated signaling

GOS may act as a TLR4 ligand, affecting downstream NF-κB and cytokine/chemokine production [12]. In **Chapter 4** and **Chapter 5**, GOS preincubation (24h) inhibited *M. haemolytica*-induced cytokine/chemokine production, TLR4 expression and MAPK and NF-κB p65 phosphorylation, as well as reduced TLR4 ligand LPS-induced cytokine/chemokine release in PBECs. In studies with mouse splenocytes and rat small intestinal epithelial cells,

preincubation with GOS for 30min or 1h inhibited LPS-induced proinflammatory responses [18, 19]. This might be related to GOS interfering with TLR4-mediated signaling, downregulating TLR4/NF-κB and secondarily MAPK pathways. Human milk oligosaccharides (HMOs), which exhibit similar anti-inflammatory effects as GOS, have been reported to inhibit TLR4 expression and signaling in mouse and piglet necrotizing colitis models and human (infant) intestinal explants, probably due to the capacity of HMOs to directly dock into the LPS-binding pocket of TLR4 [20]. Comparable to the bacterial endotoxin LPS, GOS might competitively bind to TLR4 of the lung epithelium to attenuate the initiation of the inflammatory response, thereby alleviating the airway inflammation caused by lung infections. However, caution should be taken, since modulation of inflammation is a delicate matter, and too much suppression may not benefit the host.

1.2 Anti-oxidative effect and neutralization or interference with bacterial toxins

To our knowledge **Chapter 4** is the first study to show direct evidence of the anti-oxidative effect of GOS *in vitro*. It has been reported that other non-digestible oligosaccharides (NDOs), including inulin, raffinose, and arabinoxylan-oligosaccharides, may act as reactive oxygen species (ROS) scavengers [21]. In **Chapter 4**, malondialdehyde (MDA) level (a biomarker for oxidative stress) in blood of calves was inhibited by oral GOS. This might be due to 1) GOS absorption into the systemic circulation, which might directly eliminate the ROS production from host cells (e.g., airway epithelial cells), as confirmed by the reduction of *M. haemolytica*/LPS/rotenone-induced ROS production in PBECs by GOS (**Chapter 4**) or 2) the production of glutathione S-transferases induced by short chain fatty acids (SCFAs), the fermentation products of GOS in the gut, indirectly counteract ROS *in vivo* [21]. In addition, the cytotoxicity of leukocytes caused by *M. haemolytica*-released leukotoxin is also a source of ROS in calves [22]. In **Chapter 4**, *M. haemolytica* released leukotoxin induced excessive ATP production in PBECs, which may cause the production of ROS by activating the P2X receptor (calcium influx and potassium efflux) [23]. NDOs, such as GOS and HMOs, can inhibit pathogen-induced cytotoxicity by neutralizing or interfering with released toxins [12]. Therefore, there is another possibility that GOS may indirectly inhibit the production of ROS by neutralizing or interfering with the leukotoxin released by *M. haemolytica*.

Due to the interference with TLR4-mediated signaling and anti-oxidative effect, GOS inhibited the activation of NLRP3 inflammasome *in vivo* and *in vitro*, thereby preventing the maturation and release of IL-1β (**Chapter 4**).

1.3 Anti-bacterial effect

Interestingly, some types of NDOs exhibit anti-bacterial effects [12, 24]. For example, non-sialylated HMOs directly inhibited the growth of group B *Streptococcus* [25], and pectic oligosaccharides (POS) inhibited the growth of *Clostridium perfringens* and *Bacteroides fragilis* strains *in vitro* [26]. In **Chapter 5**, we reported for the first time that GOS can not only inhibit *M. haemolytica* growth but also reduce the viability of *M. haemolytica* in a concentration-dependent manner *in vitro*. Moreover, GOS can inhibit *M. pneumoniae* growth and kill *M. pneumoniae in vitro*, which is a common pathogen related to human (childhood) respiratory infections **(Chapter 7)**. Our group also found that GOS can lower the growth of *Escherichia coli in vitro* [27]. The anti-bacterial effect of GOS might be related to the increase in bacterial membrane permeability as observed in GOS-treated *M. haemolytica in vitro*. In agreement with our findings, HMOs also have the capacity to increase the permeability of group B *Streptococcus* membranes in a concentration-dependent manner to exert anti-bacterial activity [28, 29]. Although not tested, the anti-bacterial effect of GOS on *M. pneumoniae* may also be related to the destruction of monolayer plasma membrane of *M. pneumoniae*. In addition, the effect of GOS on *M. haemolytica* and *M. pneumoniae* is concentration-dependent, concentration at 4% inhibited the *M. haemolytica* and *M. pneumoniae* growth (bacteriostatic effects), while concentrations ≥16% showed bactericidal effects.

1.4 Anti-adhesion or anti-invasive properties

Notably, although low concentrations of GOS have no significant bacteriostatic or bactericidal effects, 2% GOS prevented the adhesion to and invasion of PBECs by *M. haemolytica* (**Chapter 5**). *In vitro* studies showed that the adherence of enteropathogenic *Escherichia coli* and *Cronobacter sakazakii* to intestinal epithelial cells can be inhibited by GOS [30, 31]. It is well known that GOS can act as anti-adhesives that competitively inhibit the adhesion of pathogens to the gastrointestinal epithelium, which is mainly due to the structural similarity between GOS and bacterial binding sites/glycans of cell surface [12, 32]. Although there is no anti-adhesion study of GOS in airway/lung epithelial cells, HMOs (lacto-N-neotetraose and α2-6-sialylated lacto-N-neotetraose) have been reported to inhibit the adhesion of pneumococci to human lung epithelial cell line (A549) *in vitro* [33]. Less adhesion to and invasion of PBECs by *M. haemolytica* lead to reduced inflammatory responses and airway epithelial barrier disruption, which was showed in **Chapter 5.** Here, GOS prevented the increased cytokine/chemokine release and decreased epithelial barrier integrity caused by *M. haemolytica.*

Notably, in addition to the interference with TLR4-mediated signaling and anti-oxidative effect, the reduced *M. haemolytica* positivity and local/systemic inflammation in calves with lung infections caused by oral GOS administration in **Chapter 4** might also partly due to the direct anti-bacterial and anti-adhesion/anti-invasive effects of GOS absorbed into the system circulation.

Due to the direct effects of GOS as described above, such as interference with TLR4-mediated signaling, anti-bacterial and anti-invasive effects, GOS were innovatively applied via the intranasal route to calves with naturally occurring lung infections (**Chapter 5**). It has been described breastfeeding infants who ingest breastmilk several times per day, bathing the nasopharynx for several minutes at each feeding with a solution high in HMOs, might inhibit local bacterial adherence [34]. **Chapter 5** described that intranasal GOS partly restored the ratio of macrophages to neutrophils and inhibited the level of proinflammatory cytokines in the lungs of infected calves. In addition, local administration of GOS suppressed the number of *Pasteurellaceae* (family of *M. haemolytica*) and *M. haemolytica* positivity in the lungs, which might be related to the anti-bacterial and anti-adhesion/anti-invasive effects of GOS. Interestingly, intratracheal co-administration of HMOs (lacto-N-neotetraose and α2-6-sialylated lacto-N-neotetraose) with *pneumococci* reduced the pneumococcal CFUs in rabbit lungs and protect rabbits from bacteremia. In addition, intratracheal administration of these HMOs, 24h after infection was established, alleviated pneumonia and bacteremia. HMOs possibly act as a direct competitive inhibitor of pneumococcal adherence to lung cells [33]. In contrast, another clinical study in children found that intranasally administered HMO (3′-sialyllacto-Nneotetraose), as a prevention strategy, did not show any beneficial effects on the occurrence of acute otitis media (an upper respiratory tract infection) or the nasopharyngeal carriage of pathogens. One reason for the absence of efficacy in human beings could be inadequate exposure of the HMO to the nasopharynx due to the presence of airway mucus and the self-cleaning mechanisms [35]. In our pilot experiment, intranasal GOS was observed to be quickly eliminated from the airways, probably due to mucociliary clearance. This could be the reason why the effect of intranasal GOS on BALF composition and clinical scores in **Chapter 5** is not very pronounced. Therefore, higher dosages or more sustainable delivery of GOS at the local site, for example by use of sustained-release or delayed clearance formulations, might be a possible future step.

2. Fructo-oligosaccharides (FOS)

Like GOS, FOS also have the capacity to change the gut microbiota composition, for instance, stimulate the growth of *Bifidobacteria* and *Lactobacillus* in the

intestine, which play an important role in preventing infections and improving immune functions [12, 32]. In **Chapter 6**, although no effects on clinical scores/symptoms and *M. haemolytica* positivity were observed, FOS-fed calves showed an increased number of macrophages in the lungs and reduced severity of lung lesions, which might be related to the FOS-induced interplay between the microbiota and the immune system [14].

In addition, FOS may also have the capacity to be absorbed into the systemic circulation after oral administration and exert direct effects on host cells (e.g., airway epithelial cells), including anti-inflammatory and epithelial barrier protective effects. Eiwegger *et al*. showed *in vitro* evidence for transport of prebiotic oligosaccharides, like FOS, across the intestinal epithelial layer [36]. In **Chapter 6**, the anti-inflammatory effect of FOS in PBECs might be related to the interference with TLR5 signaling and secondarily the inhibition of NF-κB p65 and p38 MAPK phosphorylation. It has been reported that some specific HMOs, such as 2'-fucosyllactose, 6'-sialyllactose, lacto-N-neotetraose, and 3-fucosyllactose, can also suppress TLR5 signaling in HEK-Blue hTLR5 cells [37]. The accumulation of inflammatory cytokines (e.g., IL-6 and TNF-α) in the airways during lung infections is one of the important causes for the sustained impairment of the epithelial barrier function [38, 39]. Since FOS have the ability to inhibit the release of these cytokines *in vitro* and *in vivo*, the integrity of the epithelial barrier might be protected after FOS administration.

Although both FOS and GOS exert interesting anti-inflammatory properties *in vitro* and *in vivo,* different effects on immune parameters, *M. haemolytica* positivity and lung lesions are observed in calves after oral administration of these oligosaccharides (**Chapter 4** and **Chapter 6**). For example, 1% GOS restored the ratio of macrophages to neutrophils in BALF and reduced the concentration of leukocytes in blood during lung infections, while 0.25% FOS only increased the number of macrophages in BALF. A possible explanation is that the oral concentration of GOS and FOS in calves is different. Another reason might be that GOS and FOS interfere with TLR4 signaling and TLR5 signaling, respectively, while most of the *Pasteurellaceae* (e.g., *M. haemolytica*) that cause bovine lung infections express endotoxin LPS (TLR4 ligand), but not flagellin (TLR5 ligand). Although these findings are observed in human and calf airway/lung epithelial cells in **Chapter 4** and **Chapter 6**, TLRs, especially TLR4 and 5, also play an important role in the bacterial recognition and immune response of macrophages and neutrophils [40]. In addition, GOS (4%, 8% and 16%) inhibited, but FOS (1% and 2%) promoted the growth of *M. haemolytica in vitro*, which might be the reason that lower neutrophils are observed in GOS- but not FOS-fed calves. The influx of neutrophils from blood into the lungs is mainly for phagocytosis of

pathogens, while GOS-fed calves showed lower *M. haemolytica* positivity in the lungs. However, calves with oral 0.25% FOS in the early stage (experimental week 1-8) showed relative less moderate to severe lung lesions at the slaughter (week 27), compared with 1% and 2% GOS-fed calves. The increase in stool frequency and changes in stool consistency by excessive consumption and fermentation of NDOs may interfere with the absorption of nutrients [41], especially, in infected calves.

Notably, both GOS and FOS did not show clear effects on clinical scores, a scoring system for rectal temperature, coughing, nasal discharge and behavior, during the experimental period. On the one hand, it might be due to the insensitivity of clinical scores to diagnose (subclinical) lung infections compared to the parameter measurements in BALF and blood [42]; on the other hand, infections beyond the lungs (e.g., serositis, acute otitis media) might be present in the calves naturally exposed to environmental pathogens. In addition, the group antibiotic treatments shown in **Chapter 3** may also affect the scores of clinical symptoms.

3. Mixtures of galacto- and fructo-oligosaccharides

Mixtures of GOS and FOS are already widely added to dietary food or infant formulas distributed in Europe [32]. Due to the anti-inflammatory effects of GOS and FOS respectively, it is interesting to investigate the role of their mixtures in respiratory infections. However, when naturally exposed calves were fed a diet with supplemented with GOS/FOS in a ratio of 4:1 (1% GOS + 0.25% FOS), no additive effect was observed (data not shown). This might be related to the different ratios, the ratio of 9:1 was chosen to mimic the molecular size distribution and beneficial functions of HMOs in breast milk [43]. However, there is no data on using GOS/FOS to simulate bovine milk oligosaccharides in cows. The concentration of oligosaccharides in bovine milk are 0.7-1.2 g/L, which is much lower than the 12-24 g/L in breast milk [44, 45]. The identified oligosaccharides in bovine milk are around 50 types, much lower than 200 types in breast milk [46]. There are more than 70% sialylated oligosaccharides in bovine milk, in contrast, breast milk contains mainly neutral oligosaccharides, and presents approximately 10%-30% of sialylated oligosaccharides [45, 47-49]. Supplementation with additional sialylated oligosaccharides to the GOS/FOS mixture in the diet of calves with lung infections might be a possible future step, since both GOS and FOS are neutral oligosaccharides.

The *in vitro* study in **Chapter 6** showed that FOS concentrations lower than 0.25% did not exert anti-inflammatory effects, indicating that reducing the concentration of FOS may not be a better choice. In addition, increasing the total

amount of NDOs may not show better immunomodulatory effects, as observed in **Chapter 4**, calves fed with 1% GOS showed more pronounced effects on immune parameters (percentage of macrophages and neutrophils in BALF and number of leukocytes in blood) than calves fed with 2% GOS during lung infections. Moreover, feeding more NDOs may cause an increase in stool frequency and changes in stool consistency, affecting the absorption of nutrients (e.g., water) [41]. More research is needed in the future to study the possible advantage of using different ratios of GOS/FOS mixtures in respiratory infections.

Another reason for the absence of additive effect of GOS/FOS might be the degree of polymerization (DP) of FOS used in the present study. Supplementation of NDOs with different DP might be important for the inhibition of (lung) inflammation and/or infection [50]. Modulation of bifidogenic effects on the intestinal microbiota was positively correlated with DP of different NDOs [51]. The FOS in the mixtures that have been reported to be beneficial for alleviating airway diseases/inflammation are usually inulin (also called long-chain FOS, DP 2-60) [52, 53], rather than the (short-chain) FOS (DP 2-8) that was tested in **Chapter 6**. However, in these reported studies, the effects between inulin, GOS and the mixture of inulin and GOS were not compared, so it is impossible to distinguish whether the benefits of the mixture on airway diseases/inflammation are attributable to the additive effect of inulin and GOS.

Antibiotic strategy, clinical score and lung infections

In the larger calf trial in **Chapter 3-6**, individual antibiotic treatment was applied when required based on clinical signs of illness as assessed by the animal caretakers. However, the number of applied individual antibiotic treatments did not differ between the control and intervention groups ($P > 0.1$). Group antibiotic treatments were applied at week 1, 3, 6 for lung infections and week 8, 10, 12, 13 for serositis. Group antibiotic treatment was applied if 10% of the calves had been treated within 5 days, or if 5% of the calves had become ill within 24h, or when the situation required group antibiotics in the expert judgement of a veterinarian. By definition, group antibiotic treatments are equal for all treatment groups.

The rationale of this *in vivo* study was to investigate the effects of dietary supplements (e.g., NDOs) under conditions of high infection pressure. Under the inherently high infection pressure, a clear strategy of antibiotic use must be in place to prevent the calf experiment to go completely out of hand (e.g., high

mortality). Unfortunately, group antibiotic treatments cannot be avoided in the experiment, which means that for most calves at any point in time, (subclinical) lung problems might be present. This is mainly due to the difficulties in reducing antibiotic use in calf production, which is mainly related to the mingling of calves, originating from many dairy operations upon arrival at the veal farm. Therefore, antibiotic treatment may reduce the observed clinical scores, which underestimated the observed clinical infections without any antibiotic treatment.

Despite the existence of group antibiotic treatment strategies, 80% and 67% of calves still had visible lung lesions after slaughter at week 8 and 27, respectively, which reflects lung damage (**Chapter 3**). In addition, the BALF parameters at week 5 in the calves (**Chapter 3**) indicated the presence of lung inflammation (e.g., high cytokine/chemokine concentrations) and infections (e.g., number of neutrophils increased by 11 times and lymphocytes increased by more than 100 times).

Bovine respiratory disease is a complex disease that involves multiple pathogens, including viruses (e.g., bovine herpesvirus 1, bovine respiratory syncytial virus) and bacteria (e.g., *Mannheimia haemolytica*, *Histophilus somni*, *Pasteurella multocida*, *Mycoplasma bovis*) [3, 4]. In the present calf study, we measured the presence of *M. haemolytica* in the lungs to prove its relevance in our *in vivo* study and to confirm the link between the *in vitro* assays and the *in vivo* experiment. However, the current evidence does not prove that *M. haemolytica* is the main pathogenic bacteria of lung infection in calves in the current study. More microbiological diagnostics are required to analyze the respiratory pathogens in calf lungs in the future.

Statistical analyses of *in vivo* data

In the statistical analyses of *in vivo* data displayed in **Chapter 3-6**, all 6 treatments/groups were included. Subsequently, differences between specific treatments with the non-intervened control calves were tested using pre-planned contrasts and were assessed per timepoint separately. In accordance with the purpose of each chapter, we reported the p-value of the specific pre-planned contrast.

Future directions

It remains important to unravel the anti-inflammatory mechanism of NDOs (e.g., GOS and FOS) in respiratory infections. Future research should focus on the microbial changes during respiratory infections with or without NDO interventions, for example, the effects of NDOs on the dynamics of the microbiome composition in the respiratory and gastrointestinal tract during naturally occurring respiratory infections. This thesis provides possible novel treatment opportunities for respiratory infections, the intranasal GOS spray, which should be studied in more detail before being assessed in practice (in calves) or in clinical trial of humans. It will be of particular interest to investigate the changes in microbiota composition in the respiratory tract after intranasal administration of GOS in animal (calf) models to unravel the exact mechanism by which GOS exhibit anti-infective and anti-inflammatory properties. Actually, we have already planned to perform microbiome analysis in the respiratory tract (BALF) of calves after intranasal GOS administration.

We already showed that GOS have the capacity to inhibit the growth of *M. haemolytica* and even possibly kill this strain (**chapter 5**). Additionally, GOS could also inhibit the growth of and kill macrolide-resistant and macrolide-sensitive *M. pneumoniae* (human respiratory pathogen) *in vitro* (**chapter 7**). In addition to GOS, we also found that arabinoxylan can inhibit the growth of *M. haemolytica* (unpublished data). In the next step, we will also investigate the effect of arabinoxylan on the growth of macrolide-resistant and macrolide-sensitive *M. pneumoniae*. The anti-bacterial mechanism of GOS and arabinoxylan is still unclear. In the future, it may be interesting to study the effect of these anti-bacterial carbohydrates on bacterial glycocalyx, bacterial membrane formation and nutrient uptake. In addition, more carbohydrates should be included to study their (structure-related) anti-bacterial mechanisms. Furthermore, to increase our knowledge about the strain-specificity of GOS and other carbohydrates, more (human) pathogens should be involved in future research. Perhaps, it will be possible to design anti-pathogenic carbohydrates with corresponding structures based on different (drug-resistant) strains.

Besides the effect of NDOs on pathogenic bacteria as described above, the effect of NDOs on the commensal bacteria in the gut and airways during respiratory infections might also be interesting. It has been proven that the growth of commensal microbiota (e.g., *Bifidobacteria* and *Lactobacilli*) can inhibit the presence of pathogenic bacteria in the gastrointestinal and respiratory tract, which may be due to their competition for nutrition [12, 54]. Due to the intervention of NDOs, the balance of nutritional competition may be biased towards the

commensal microbiota. Excitingly, our group started to investigate the effects of NDO intervention (orally) on airway diseases, such as asthma, chronic obstructive pulmonary disease (COPD), etc. years ago, and now for the first time showed that intranasal NDO administration might be the possible future strategy to inhibit respiratory infections.

Another interesting direction may be microbiota transplantation in calves with respiratory infections. We already conducted a new experiment with oral and rectal administration of microbiota with or without GOS feeding in calves with naturally occurring respiratory infections. In addition, intranasal or intratracheal transplantation of respiratory microbiota with or without NDOs in calves with respiratory infections may also be a challenging future direction. It has been reported that intranasal administration of *Lactobacillus* strains originating from the nasopharyngeal microbiota of calves reduced nasal colonization by *M. haemolytica* and the relative abundance of *M. haemolytica* in the trachea, even though these calves inoculated with *M. haemolytica* in the reported study did not develop respiratory infections [54]. Our goal is that the transplantation of microbiota can be applied to calves, humans and other animals suffering from respiratory infections in the future.

In addition to respiratory infections, the effects of NDOs with or without microbiota transplantation on other (respiratory) diseases, such as asthma, COPD, cancer, and skin infections, may also be an interesting topic for future research. Gluco-oligosaccharides have been reported to be applied topically in a cream to clear adherent *Staphylococcus aureus* from skin lesions of atopic dermatitis [55]. Using NDOs instead of antibiotics to treat skin infections may contribute to the remission of antimicrobial resistance.

Our group has demonstrated that oral GOS or the mixtures of FOS and *Bifidobacteria* decreased airway eosinophilia and allergic inflammation in a murine asthma model [13, 56]. It is still not clearly described whether these chronic respiratory diseases (e.g., asthma, COPD) can cause changes in the respiratory microbiota. Perhaps, intranasal or intratracheal transplantation of respiratory microbiota will be the alternative for these respiratory diseases that lack effective treatments.

The interaction between NDOs and TLRs is also a direction worthy of in-depth study. Although the effect of GOS on TLR4 signaling and FOS on TLR5 signaling was investigated *in vitro*, it could not be verified in calves due to the lack of genetic tools. We only measured the protein expression of TLR4 and TLR5 *in vitro*, which is insufficient to illustrate the activation of TLR4 or TLR5 and to proof that the GOS and FOS act via TLR4 and TLR5 signaling. Therefore, verification in rodents

may be an alternative in the future, including the use of knockout mice and intervention with TLR inhibitors. Cellular gene-editing, for example, knock-out or knock-down of TLR4 or TLR5 *in vitro* prior to GOS or FOS incubations will provide another step forward. Moreover, except TLR4 and TLR5, the interaction of NDOs with other TLRs (e.g., TLR1, TLR2, TLR6) should be studied.

In this thesis, NDOs are administered orally or intranasally in calves, but the metabolic distribution of NDOs in the systemic circulation is not clear. Therefore, the presence of NDOs in blood and urine of calves, as well as the metabolic kinetics of NDOs should be measured. Moreover, it is already feasible and safe to supply oligosaccharides (such as αGal-oligosaccharides, glucose-oligosaccharides, and hyaluronic acid-derived oligosaccharides) during intravenous infusion in humans [57] and animals (e.g., mice, pigs, baboons) to provide energy source or treat diseases [58-60]. This indicated the future possibility of intravenous application of GOS or FOS after a safety evaluation in humans and animals with respiratory diseases. In addition, the direct effects of NDOs on host cells, other than airway epithelial cells, including macrophages and neutrophils, are also valuable directions for future research.

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Summary

Summary

The studies described in this thesis were performed to gain more insight into the potential benefits of non-digestible oligosaccharides (NDOs), including galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), and their mixtures, in respiratory infections. Here, we summarize the most important findings of this thesis, including establishing animal models of respiratory infections *ex vivo* and *in vivo*, possible working mechanisms of GOS and FOS, and related benefits for human (respiratory) health.

Modelling Respiratory Infections *ex vivo* and *in vivo*

Cellular and animal models are essential for the characterization of the pathophysiology of diseases, like human respiratory infections, and for the evaluation of novel therapeutic agents. Therefore, *ex vivo* and *in vivo* models of respiratory infections were developed. In **Chapter 2**, an optimized culture method for primary bronchial epithelial cells (PBECs) obtained from calves was established and *Mannheimia haemolytica* and lipopolysaccharides (LPS) - induced inflammatory responses in PBECs were detected. *M. haemolytica* and LPS significantly increased cytokine and chemokine release and decreased epithelial integrity in PBECs, possibly by activating "Toll-like receptor (TLR) - mediated NF-κB and MAPKs" signaling pathways. In **Chapter 3**, calves were naturally exposed to environmental pathogens and developed lung infections, which is generally observed in the veal calf industry. These calves demonstrated recruitment of neutrophils, depletion of macrophages and production of inflammatory cytokines and chemokines during lung infections; these parameters can contribute to the diagnosis of bovine respiratory diseases. The rise of antimicrobial-resistant pathogens and the economic loss of preventing antimicrobial resistance highlight the importance of new intervention strategies.

Pathogenesis and Early Life Intervention of Respiratory Infections

GOS and FOS were investigated in the *ex vivo* and *in vivo* models of respiratory infections. GOS and/or FOS were orally or intranasally administrated to calves with naturally occurring respiratory infections. Moreover, PBECs were treated with GOS or FOS prior to and during *M. haemolytica* and LPS stimulation.

In **Chapter 4**, although no effects on clinical scores were observed, orally administered GOS alleviated lung infections and decreased the inflammatory responses (airways and blood) in the early stage of infected calves, which might

be partly due to the inhibited activation of NLR family pyrin domain containing 3 (NLRP3) inflammasome and subsequently reduced maturation and release of interleukin (IL)-1β in the lung tissue. Inhibition of NLRP3 inflammasome activation and IL-1β release has also been observed in PBECs stimulated with *M. haemolytica* and LPS and pretreated with GOS. Interference with TLR4-mediated proinflammatory signal transduction pathways and inhibition of reactive oxygen species (ROS) and ATP production by GOS were also observed related to this NLRP3 inflammasome pathway. In **Chapter 5,** intranasally administered GOS showed no effects on clinical scores but reduced the inflammatory response and pathogen burden (decreased *Pasteurellaceae* number and *M. haemolytica* positivity) in the lungs of infected calves during early life, which might be partly related to a GOS-induced decrease in adhesion to and invasion of airway epithelial cells by *M. haemolytica* or GOS might even directly kill *M. haemolytica*. In addition, reduced adhesion and invasion also prevented the destruction of the airway epithelial barrier. In **Chapter 6**, orally administered FOS showed no effects on clinical scores and *M. haemolytica* positivity as well as exerted limited effects on immune parameters but decreased the severity of lung lesions and inflammatory responses (airways and blood) in infected calves, which might be partly related to the FOS-induced prevention of *M. haemolytica*-induced airway epithelial barrier dysfunction. The interference of FOS with TLR5-mediated signaling pathways may be involved as well.

Relevance with Human Respiratory Infections and Health

The relevance for the human situation was explored by using human respiratory pathogens for investigating the beneficial effects of dietary fiber. In **Chapter 7**, the growth of macrolide-resistant and macrolide-sensitive *Mycoplasma pneumoniae*, a common pathogen related to human (childhood) respiratory infections, was lowered by GOS and FOS, while particularly GOS killed the vast majority of macrolide-resistant and macrolide-sensitive *M. pneumoniae in vitro*. In **Chapter 9**, a broader view on microbiota-dependent and -independent effects of dietary fiber, such as NDOs, on human health is presented. This review serves as a supplement to our findings and summaries the benefits and potential mechanisms of dietary fiber on human health.

The above-mentioned pathogenesis of lung infections and directly protective effects of GOS/FOS are summarized in the following figure:

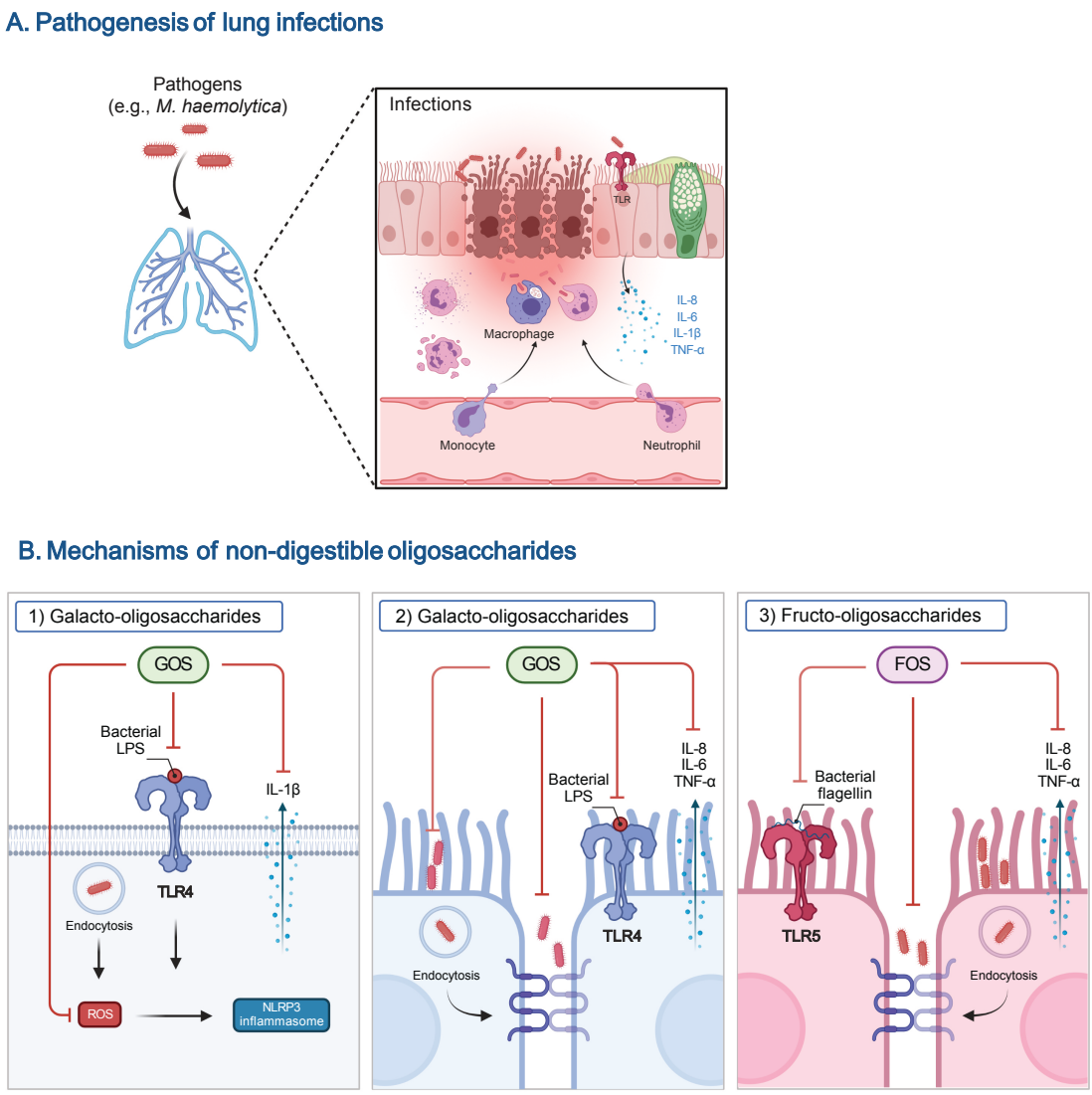


Figure 1. The pathogenesis of lung infections and the postulated mechanisms of non-digestible oligosaccharides. (A) During lung infections, pathogens (e.g., *M. haemolytica*) induce the release of proinflammatory mediators (e.g., IL-8, IL-6, IL-1β, and TNF-α) by activating TLR signaling to recruit immune cells (e.g., neutrophils, macrophages), contributing to the phagocytosis of pathogens and elimination of inflammation in the early stage of infections. However, the impairment of the airway epithelial barrier, the accumulation of proinflammatory mediators, the depletion of macrophages, and the infiltration of neutrophils caused by excessive pathogens and their released virulence factors (e.g., LPS), lead to lung injury and organ dysfunction and even death of susceptible hosts (**Chapter 2** and **Chapter 3**). **(B)** 1) The anti-inflammatory mechanisms of GOS may include the inhibition of NLRP3 inflammasome activation via the interference with TLR4 signaling and the decrease of ROS production, subsequently reducing IL-1β release (**Chapter 4**), and 2) the decrease in adhesion to and invasion of

airway epithelial cells by pathogens or the possibly direct killing of pathogens induced by GOS (**Chapter 5** and **Chapter 7**). 3) Anti-inflammatory effects of FOS may be related to the interference with TLR5 proinflammatory signaling and protection of airway epithelial barrier function (**Chapter 6**). *FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides; IL, interleukin; LPS, lipopolysaccharides; NLRP3, NLR family pyrin domain containing 3; ROS, reactive oxygen species; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α.*

Conclusions

The studies described in this thesis have investigated the anti-inflammatory effects and possible mechanisms of non-digestible oligosaccharides (NDOs), including galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), in respiratory infections in calves naturally exposed to respiratory pathogens, with a possible translation to human respiratory infections. A naturally occurring lung infection model in calves, *M. haemolytica*/LPS/flagellin-treated primary bronchial epithelial cells obtained from calves, and LPS/flagellin-treated human lung epithelial cells were used to investigate the potential effects and mechanisms of GOS and FOS. Anti-inflammatory effects of GOS and FOS observed *in vitro* were compared with the effects on inflammatory markers in airway and blood of calves fed with these NDOs. The main mechanisms related to anti-inflammatory effects of GOS and FOS derived from the present *in vitro* studies include 1) the anti-bacterial effect of GOS at high concentrations (8% and 16%) on respiratory pathogens (e.g., *M. haemolytica* and *M. pneumoniae*), 2) interference with TLR4 proinflammatory signaling and inhibition of oxidative stress and *M. haemolytica* adhesion to and invasion of host cells by GOS at low concentrations (2%), and 3) interference with TLR5 proinflammatory signaling and protection of airway epithelial barrier function by 0.5% FOS. Due to these mechanisms, (oral or intranasal) GOS and FOS (partly) relieved lung infections and suppressed airway and systemic inflammation in calves. In addition, the changes in microbiota composition in the respiratory and gastrointestinal tract caused by GOS and/or FOS (orally or intranasally) during respiratory infections in calves might also play an important role in the observed *in vivo* effects. Finally, these results might contribute to reducing the future use of antibiotics in livestock species and humans and need to be incorporated into the evolving knowledge of microbiota-dependent or -independent effects of NDOs.

Major findings in this thesis:

1. *M. haemolytica* and lipopolysaccharides significantly increased cytokine and chemokine release and decreased epithelial integrity in primary bronchial epithelial cells, possibly by activating "TLR-mediated NF-κB and MAPKs" signaling pathways.
2. The recruitment of neutrophils, depletion of macrophages and production of inflammatory cytokines and chemokines were observed in the lungs of calves during natural occurring respiratory infections.
3. Oral GOS alleviated the lung infections and decreased the inflammatory responses (airways and blood) in calves, which might be partly due to the interference withTLR4 signaling and the inhibition of NLRP3 inflammasome activation, subsequently reducing IL-1β release.
4. Intranasal GOS reduced the inflammatory response and pathogen burden in the lungs, which might be partly related to a GOS-induced decrease in adhesion to and invasion of airway epithelial cells by *M. haemolytica* or the possibly bactericidal effect of GOS.
5. Oral FOS decreased the severity of lung infections and inflammatory responses (airways and blood) in calves, which might be partly related to the interference with TLR5 signaling and the prevention of airway epithelial barrier dysfunction.
6. The growth of macrolide-resistant and macrolide-sensitive *M. pneumoniae* was lowered by GOS and FOS, while particularly GOS killed the vast majority of these pathogens *in vitro*.
7. The anti-inflammatory effects of GOS and FOS observed *in vitro* and *ex vivo* validated the reduction of airway and systemic inflammation in calves naturally exposed to environmental pathogens.
8. Although the effects of these oligosaccharides on clinical scores and performance were less strong or even absent, this confirms the importance of further research concerning the role of NDOs in reducing antimicrobial use and resistance.

Chapter X

Appendix

Nederlandse Samenvatting

De studies beschreven in dit proefschrift zijn uitgevoerd om meer inzicht te krijgen in de mogelijke voordelen van niet-verteerbare oligosachariden zoals galacto-oligosachariden (GOS) and fructo-oligosachariden (FOS) en mengsels hiervan op luchtwegaandoeningen. In dit hoofdstuk worden de meest belangrijke bevindingen van dit proefschrift samengevat, waaronder optimalisering van (dier)modellen voor luchtwegaandoeningen *ex vivo* en *in vivo*, mogelijke werkingsmechanismen van GOS en FOS en mogelijke toepassingen voor de humane (long)gezondheid.

Modelleren van luchtweginfecties *ex vivo* en *in vivo*

Cellulaire en diermodellen zijn essentieel voor de karakterisering van de pathofysiologie van ziekten zoals luchtweginfecties bij de mens en voor de evaluatie van nieuwe therapeutische middelen. Gedurende dit PhD project werden er *ex vivo* en *in vivo* modellen van luchtweginfecties ontwikkeld. In **hoofdstuk 2** werd een kweekmethode voor primaire bronchiale epitheelcellen (PBECs) uit kalveren geoptimaliseerd. In dit model werden er ontstekingsreacties veroorzaakt indien de cellen met pathogenen zoals *M. haemolytica* of met lipopolysacharide (LPS), een bestanddeel van de buitenmembraan van Gram-negatieve bacteriën, geïncubeerd werden. *M. haemolytica* en LPS verhoogden de afgifte van cytokines en chemokines en verminderden de epitheliale barrière functie van de PBECs mogelijk door het activeren van Toll-like receptors (TLRs) die een rol spelen in het immuunsysteem. In **hoofdstuk 3** werden kalveren van nature blootgesteld aan ziekteverwekkers uit de omgeving en ontwikkelden de kalveren longinfecties, een fenomeen dat algemeen wordt waargenomen in de kalverhouderij. De kalveren vertoonden een verhoging van het aantal neutrofielen, een vermindering van het aantal macrofagen en een geremde productie van inflammatoire cytokines en chemokines in de luchtwegen tijdens deze longinfecties. Deze parameters kunnen bijdragen aan de diagnose van luchtwegaandoeningen bij runderen. De toename van antimicrobiële resistente pathogenen en het economische verlies gerelateerd aan antimicrobiële resistentie benadrukken het belang van nieuwe interventiestrategieën.

Pathogenese en vroegtijdige interventie van luchtweginfecties

De werking van GOS en FOS werd onderzocht in de *ex vivo* en *in vivo* modellen van luchtweginfecties. GOS en/of FOS werden oraal of intra-nasaal toegediend

aan kalveren met van nature voorkomende luchtwegpathogenen. Daarnaast werden PBECs vóór en tijdens *M. haemolytica*- en LPS-stimulatie behandeld met GOS of FOS.

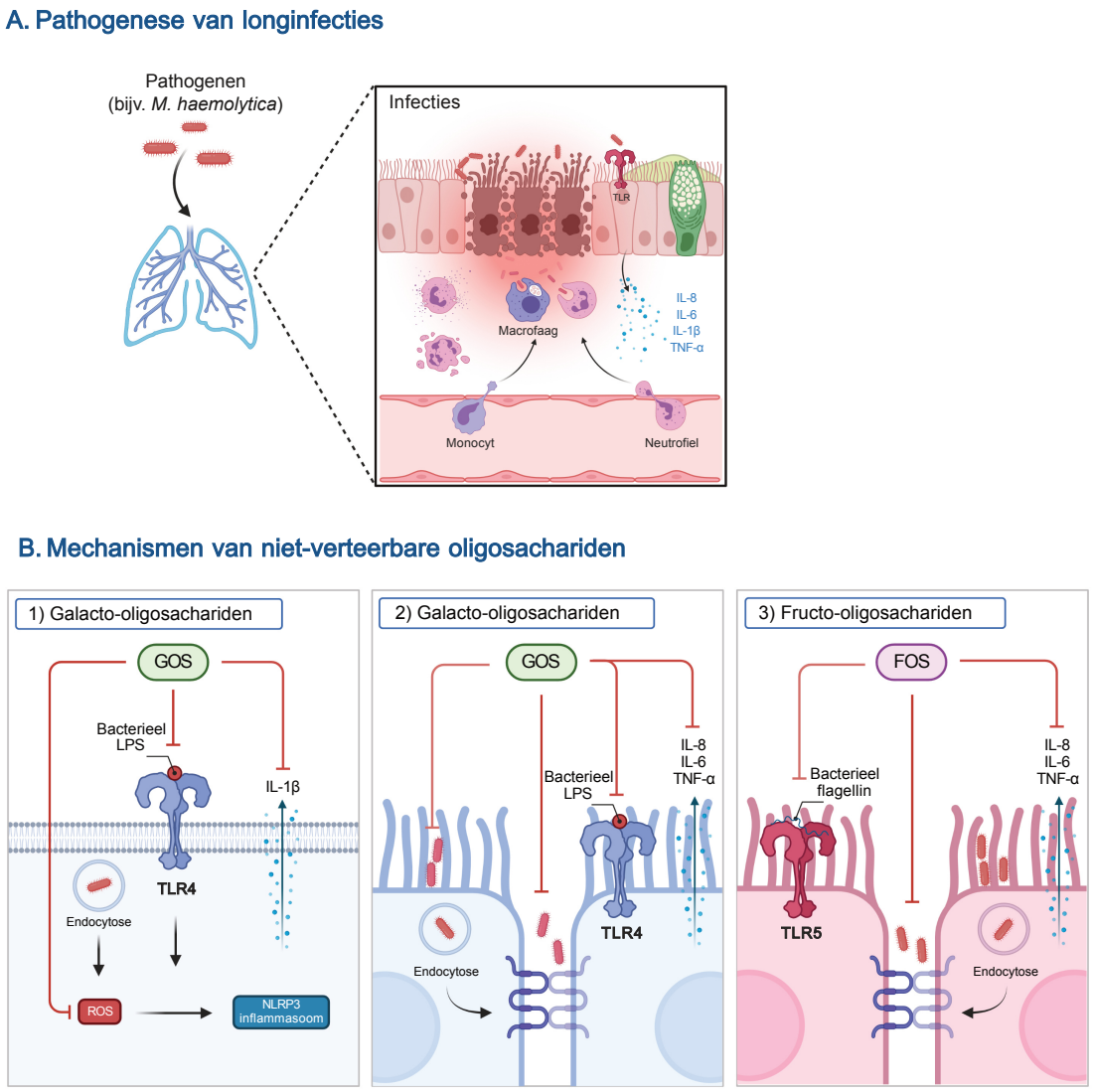
In **hoofdstuk 4** werden geen effecten op klinische scores waargenomen, maar oraal toegediend GOS verminderde de longinfecties en ontstekingsreacties (in luchtwegen en bloed) in het vroege leven van geïnfecteerde kalveren. Dit zou gedeeltelijk te wijten kunnen zijn aan de geremde activering van het eiwit NLRP3-inflammasoom, dat verantwoordelijk is voor het mediëren van de ontstekingsreactie, en vervolgens verminderde productie en afgifte van IL-1β in het longweefsel. Dit is ook waargenomen in PBECs die werden gestimuleerd met *M. haemolytica* en LPS en voorbehandeld waren met GOS. Stimulatie van TLR4-gemedieerde pro-inflammatoire signaaltransductieroutes, remming van oxidatieve stress en remming van ATP productie door GOS werden ook waargenomen in verband met deze NLRP3-inflammasoomroute. In **hoofdstuk 5** liet GOS, toegediend via de intra-nasale route, geen effecten zien op klinische scores, maar verminderde GOS de ontstekingsreactie en de ziekteverwekker (verlaagd *Pasteurellaceae*-aantal en *M. haemolytica*-positiviteit) in de longen van geïnfecteerde kalveren. GOS zou mogelijk de adhesie van *M. haemolytica* aan luchtweg-/longepitheelcellen of de invasie van *M. haemolytica* kunnen remmen. Er zijn zelf indicaties dat GOS *M. haemolytica* direct zou kunnen doden. Bovendien zorgden de verminderde adhesie en invasie ook voor de beschadiging van de epitheliale barrière van de luchtwegen. In **hoofdstuk 6** vertoonde FOS in het dieet geen effecten op klinische scores en de aanwezigheid van *M. haemolytica*. Ook werden er beperkte effecten op immuunparameters gevonden, maar FOS verminderde wel de ernst van longlaesies en ontstekingsreacties (luchtwegen en bloed) bij geïnfecteerde kalveren. Dit kan gedeeltelijk gerelateerd zijn aan de mogelijkheid dat FOS de luchtwegepitheelbarrière kan versterken en daardoor de effecten van *M. haemolytica* kan voorkomen. Het stimuleren van TLR5-gemedieerde signaalroutes door FOS kan ook een rol spelen.

Relevantie voor menselijke luchtweginfecties en gezondheid

De relevantie voor de menselijke situatie werd onderzocht door voor mensen respiratoire pathogenen te gebruiken om de gunstige effecten van de beschreven oligosachariden te onderzoeken. In **hoofdstuk 7** werd de groei van macrolide-resistente en macrolide-gevoelige *M. pneumoniae*, een veel voorkomend pathogeen gerelateerd aan humane (kinder)luchtweginfecties, geremd door GOS en FOS. Met name GOS doodde zowel de macrolide-resistente als macrolide-gevoelige *M. pneumoniae in vitro*. In **hoofdstuk 9** wordt een bredere kijk op

microbiota-afhankelijke en -onafhankelijke effecten van voedingsvezels, zoals niet-verteerbare oligosachariden, op de menselijke gezondheid gepresenteerd. Dit review dient als een aanvulling op onze bevindingen en vat de voordelen en mogelijke mechanismen van voedingsvezels voor de menselijke gezondheid samen.

De bovenvermelde pathogenese van luchtweginfecties en de direct beschermende effecten van GOS en FOS zijn samengevat in de volgende figuur:



Figuur 1. De pathogenese van luchtweginfecties en de veronderstelde mechanismen van niet-verteerbare oligosachariden. (A) Tijdens infecties induceren pathogenen (bijv. *M. haemolytica*) de afgifte van pro-inflammatoire mediatoren (bijv. IL-8, IL-6, IL-1β en TNF-α) via activatie van TLR-signaalroutes. Dit zal leiden tot het rekruteren van immuuncellen (bijv. neutrofielen en macrofagen) die bijdragen aan de

fagocytose van pathogenen en eliminatie van ontstekingen in het vroege stadium van infecties. De vermindering van de epitheliale luchtwegbarrière, de accumulatie van pro-inflammatoire mediatoren, de vermindering van het aantal macrofagen en de infiltratie van neutrofielen veroorzaakt door een grote hoeveelheid pathogenen en hun vrijgegeven virulentiefactoren (bijv. LPS), leiden tot longbeschadiging en orgaandysfunctie en zelfs de dood van gevoelige gastheren (**hoofdstuk 2** en **hoofdstuk 3**). (B) Niet-verteerbare oligosachariden kunnen dit op drie mogelijke manieren beïnvloeden: 1) De ontstekingsremmende mechanismen van GOS kunnen het activeren van NLRP3-inflammasoom mogelijk remmen via de activering van TLR4 signalering en de afname van oxidatieve stress, waardoor de IL-1β afgifte wordt verminderd (**hoofdstuk 4**), 2) GOS zou mogelijkerwijs de afname van adhesie aan luchtwegepitheelcellen en invasie van pathogenen kunnen beïnvloeden, of zou zelfs de pathogenen direct kunnen doden (**hoofdstuk 5** en **hoofdstuk 7**) en 3) Ontstekingsremmende effecten van FOS zouden verband kunnen houden met de activatie van TLR5 pro-inflammatoire signaal-transductie routes en de bescherming van de epitheliale barrièrefunctie van de luchtwegen (**hoofdstuk 6**). *FOS, fructo-oligosachariden; GOS, galacto-oligosachariden; IL, interleukine; LPS, lipopolysachariden; NLRP3, NLR familie pyrine domein met 3; ROS, reactieve zuurstofspecies; TLR, Toll-like receptor; TNF-α, tumor necrose factor-α.*

Conclusies

Met de studies beschreven in dit proefschrift zijn de ontstekingsremmende effecten en mogelijke mechanismen onderzocht van niet-verteerbare oligosachariden, waaronder GOS en FOS, bij luchtweginfecties in kalveren die van nature zijn blootgesteld aan respiratoire pathogenen. Hierbij hebben we een mogelijke vertaalslag gemaakt naar luchtweginfecties bij de mens. Een natuurlijk voorkomend longinfectiemodel bij kalveren, primaire bronchiale epitheelcellen verkregen van kalveren en humane longepitheelcellen werden gebruikt om de mogelijke effecten en mechanismen van GOS en FOS te onderzoeken. *In vitro* waargenomen ontstekingsremmende effecten van GOS en FOS werden vergeleken met de effecten op ontstekingsmarkers in luchtwegen en bloed van kalveren die met deze niet-verteerbare oligosachariden waren gevoerd. De belangrijkste mechanismen in verband met de ontstekingsremmende effecten van GOS en FOS die uit de huidige *in vitro* experimenten naar voren komen, zijn 1) het bacteriedodende effect van GOS in hoge concentraties (8% en 16%) op respiratoire pathogenen (bijv. *M. haemolytica* en *M. pneumoniae*), 2) de activatie van TLR4 pro-inflammatoire signaal-transductie routes, de remming van oxidatieve stress en de remming van de invasie en de adhesie van *M. haemolytica* aan luchtwegepitheelcellen door GOS bij lage concentraties (2%) en 3) de activatie van TLR5 pro-inflammatoire signaal-transductie routes en de bescherming van de epitheliale luchtwegbarrière door 0.5% FOS. Door deze mechanismen verminderden (oraal of intranasaal toegediend) GOS en FOS (gedeeltelijk) longinfecties en onderdrukten luchtweg- en systemische ontstekingen bij kalveren. Daarnaast kunnen de veranderingen in de samenstelling van de microbiota in de luchtwegen en het maagdarmkanaal veroorzaakt door GOS en/of FOS (oraal of intranasaal) tijdens luchtweginfecties bij kalveren ook een belangrijke rol spelen in de waargenomen *in vivo* effecten. Ten slotte kunnen de resultaten beschreven in dit proefschrift mogelijk bijdragen aan het verminderen van het gebruik van antibiotica bij diersoorten en mensen in de toekomst en dragen ze bij aan de groeiende kennis over microbiota-afhankelijke of -onafhankelijke effecten van NDO's.

Belangrijkste bevindingen in dit proefschrift:

1. *M. haemolytica* en lipopolysacchariden verhoogden de afgifte van cytokines en chemokines en verminderden de luchtwegbarrièrefunctie in primaire bronchiale epitheelcellen, mogelijk door het activeren van TLR-signaal-transductie routes.
2. De rekrutering van neutrofielen, vermindering van het aantal macrofagen en productie van inflammatoire cytokines en chemokines werden waargenomen in de longen van kalveren tijdens natuurlijk voorkomende luchtweginfecties.
3. GOS in het dieet verminderde de luchtweginfecties en verlaagde de ontstekingsreacties (in luchtwegen en bloed) bij kalveren, wat gedeeltelijk veroozaakt kan zijn door de activatie van TLR4-signalering en de remming van NLRP3-inflammasoom activatie waardoor de IL-1β afgifte wordt verminderd.
4. GOS toegediend via de neus verminderde de ontstekingsreacties en pathogene belasting in de longen, wat gedeeltelijk verband zou kunnen houden met een door GOS geïnduceerde afname van adhesie aan luchtwegepitheelcellen en invasie van *M. haemolytica* of het bacteriedodende effect van GOS.
5. Oraal toegediend FOS verminderde de ernst van longinfecties en ontstekingsreacties (in luchtwegen en bloed) bij kalveren, wat gedeeltelijk veroorzaakt kan zijn door de activering van TLR5-signalering en het versterken van de luchtwegepitheelbarrière.
6. De groei van macrolide-resistente en macrolide-gevoelige *M. pneumoniae* werd geremd door GOS en FOS, terwijl met name GOS deze pathogenen *in vitro* doodde.
7. De *in vitro* en *ex vivo* waargenomen ontstekingsremmende effecten van GOS en FOS werden bevestigd door de vermindering van luchtweg en systemische ontstekingen bij kalveren die van nature zijn blootgesteld aan pathogenen uit de omgeving.
8. Hoewel de effecten van deze oligosachariden op klinische scores en prestaties minder sterk of afwezig waren, bevestigen de resultaten in dit proefschrift het belang van verder onderzoek naar de rol van niet-verteerbare oligosachariden bij het terugdringen van het gebruik van antibiotica en de ontwikkeling van resistentie.

中文总结

总结

本论文中描述的研究旨在更深入地了解非消化性低聚糖（包括低聚半乳糖、低聚果糖及其混合物）在呼吸道感染中的潜在益处。在这里，我们总结了论文中最重要的发现，包括体外和体内呼吸道感染动物模型的建立、低聚半乳糖和低聚果糖可能的工作机制以及这些非消化性低聚糖对人类（呼吸）健康的益处。

体外和体内呼吸道感染动物模型的建立

细胞和动物模型对于认知疾病（如人类呼吸道感染）的病理生理学特征以及评估新型治疗方法至关重要。因此，我们开发了呼吸道感染的离体和体内模型。在第 2 章中，我们建立和优化了小牛原代支气管上皮细胞的分离和培养方法，并且检测了该支气管上皮细胞针对脂多糖和溶血性曼海姆氏菌的炎症反应。脂多糖和溶血性曼海姆氏菌显著增加了该支气管上皮细胞中细胞因子和趋化因子的释放并降低了该细胞的上皮完整性，这可能是因为“TLR 介导的 NF-κB 和 MAPKs”信号通路的激活。在第 3 章中，小牛自然暴露于环境病原体并发生肺部感染，这在小牛犊养殖业中很常见。这些小牛在感染期间肺部表现出了中性粒细胞的募集、巨噬细胞的耗竭以及炎性细胞因子和趋化因子的产生，这些参数有助于牛呼吸道疾病的诊断。而抗菌药物耐药型病原体的增加和为预防抗菌药物耐药性所产生的经济损失凸显了新干预策略的重要性。

呼吸道感染的发病机制和早期干预

在呼吸道感染的离体和体内模型中研究了低聚半乳糖和低聚果糖的效果。患有呼吸道感染的小牛接受低聚半乳糖或低聚果糖的口服或鼻腔给药。此外，原代支气管上皮细胞在脂多糖和溶血性曼海姆氏菌刺激前接受低聚半乳糖或低聚果糖的预处理。

在第 4 章中，虽然对临床评分的影响有限，但口服低聚半乳糖在实验的早期阶段减轻了小牛的肺部感染并减少了气道和血液中的炎症反应，这可能部分是由于 NLRP3 炎症小体的激活受到了抑制，随后降低了肺组织中白介素 1β的成熟和释放。在体外，低聚半乳糖预处理也抑制了脂多糖和溶血性曼海姆氏菌引起的原代支气管上皮细胞中 NLRP3 炎症小体的激活。低聚半乳糖对 NLRP3 炎症小体激活的抑制和其对 TLR4 介导的促炎信号转导的干扰以及对活性氧和 ATP 产生的抑制有关。在第 5 章中，鼻内给予低聚半乳糖对小牛临床评分没有影响，但降低了感染

早期肺部的炎症反应和病原体负荷(即,巴斯德氏菌数量和溶血性曼海姆氏菌阳性率的减少),这可能部分是由于低聚半乳糖降低了溶血性曼海姆氏菌对气道上皮细胞的粘附和侵袭,甚至可能是由于低聚半乳糖直接杀死了溶血性曼海姆氏菌。此外,溶血性曼海姆氏菌对气道上皮细胞粘附和侵袭的减少也可以阻止气道上皮屏障的破坏。在第 6 章中,虽然口服低聚果糖对感染小牛的临床评分和溶血性曼海姆氏菌阳性率没有影响,且对免疫学参数的影响也有限,但是降低了小牛肺部病变的严重程度,并且抑制了气道以及血液中的炎症反应,这可能部分是由于低聚果糖预防了溶血性曼海姆氏菌诱导的气道上皮屏障功能障碍。此外,低聚果糖对 TLR5 介导的信号通路的干扰也可能参与其中。

与人类呼吸道感染和健康的相关性

通过利用在人呼吸道病原体中的研究来探索膳食纤维对人类健康的益处。在第 7 章中,低聚半乳糖和低聚果糖抑制了大环内酯类抗生素耐药型和敏感型肺炎支原体的生长,其中肺炎支原体是一种引起人类(儿童)呼吸道感染的常见病原体,而特别的是,低聚半乳糖在体外可以同时杀死耐药型和敏感型肺炎支原体。在第 9 章中,综述了膳食纤维(例如,非消化性低聚糖)对人类健康的菌群依赖性和非依赖性影响。该综述作为我们研究发现的补充,总结了膳食纤维对人类健康的益处和潜在机制。

结论

本论文中描述的研究调查了非消化性低聚糖,包括低聚半乳糖和低聚果糖,在小牛自然发生的呼吸道感染中的抗炎作用和可能机制,这些作用和机制可能会转化为人类呼吸道感染研究的基础。使用了小牛自然发生的肺部感染模型和溶血性曼海姆氏菌、脂多糖、鞭毛蛋白刺激的原代支气管上皮细胞模型以及脂多糖、鞭毛蛋白刺激的人气道上皮细胞模型来研究低聚半乳糖和低聚果糖的效果和潜在机制。将体外模型中观察到的低聚半乳糖和低聚果糖的抗炎作用与这些非消化性低聚糖对小牛气道和血液中炎症标志物的影响进行了比较。目前体外研究得出的低聚半乳糖和低聚果糖抗炎作用相关的机制主要包括:1) 高浓度(8%和 16%)的低聚半乳糖对呼吸道病原体(例如,溶血性曼海姆氏菌和肺炎支原体)的杀菌作用;2) 低浓度(2%)的低聚半乳糖对 TLR4 促炎信号和氧化应激的抑制以及对溶血性曼海姆氏菌粘附和侵袭宿主细胞的遏制;3)0.5%浓度的低聚果糖对 TLR5 促炎信号的

干扰和对气道上皮屏障功能的保护。由于这些有趣的机制，（口服或鼻内给药）低聚半乳糖或低聚果糖（部分）减轻了小牛的肺部感染并抑制了气道和全身的炎症。此外，在小牛呼吸道感染期间由于低聚半乳糖或低聚果糖（口服或鼻内给药）引起的呼吸道和胃肠道菌群组成的变化也可能在这些体内结果中发挥重要作用。最后，这些结果可能有助于减少抗生素未来在牲畜和人类中的使用，并且需要纳入到非消化性低聚糖菌群依赖性和非依赖性影响的不断发展的知识中去。

本论文的主要发现：

1. 溶血性曼海姆氏菌和脂多糖显著增加了原代支气管上皮细胞细胞因子和趋化因子的释放，并降低了上皮完整性，这可能是由于“TLR 介导的 NF-κB 和 MAPKs”信号通路的激活。
2. 自然发生的呼吸道感染期间，在小牛的肺中观察到了中性粒细胞的募集、巨噬细胞的耗竭以及炎性细胞因子和趋化因子的产生。
3. 口服低聚半乳糖减轻了小牛的肺部感染并减弱了气道和血液中的炎症反应，这可能部分是由于 TLR4 信号传导的干扰和 NLRP3 炎性小体激活的抑制，从而导致了白介素 1β释放的减少。
4. 鼻内给予低聚半乳糖降低了肺部的炎症反应和病原体负荷，这可能部分与低聚半乳糖引起的溶血性曼海姆氏菌对气道上皮细胞粘附和侵袭的减少有关，此外还可能与低聚半乳糖的杀菌作用有关。
5. 口服低聚果糖降低了小牛肺部感染的严重程度以及气道和血液中的炎症反应，这可能部分与 TLR5 信号传导的干扰和气道上皮屏障功能的保护有关。
6. 低聚半乳糖和低聚果糖抑制了大环内酯类抗生素耐药型和敏感型肺炎支原体的生长，尤其低聚半乳糖在体外杀死了肺炎支原体。
7. 在体外和体外观察到的低聚半乳糖和低聚果糖的抗炎作用证实了自然暴露于环境病原体的小牛的气道和全身炎症的减少。
8. 尽管这些低聚糖对小牛临床评分和临床表现的影响不那么强烈甚至不存在，但这证明了进一步研究非消化性低聚糖在减少抗菌药物使用和耐药性中的作用的重要性。

Acknowledgements

Acknowledgements

Before I walked out of the forest, I saw the light of the sunrise, which was the end of my PhD project and the beginning of my new life. PhD research is interesting, as fresh as forest exploration but also full of challenges. Now, I would like to especially thank some people who helped and supported me during this exciting exploration.

Promoters

Prof. dr. G. Folkerts, dear Gert, I can finally share my admiration and gratitude for you as a supervisor. Since I started working at UIPS in 2017, I have regarded you as my role model at work. You provided me with very important recommendations and brought me interesting collaborations during my project. Because of you, my project was successful and fruitful. Your friendly manner, warm personality and scientific insight were often what I needed, and I learned a lot from your positive attitude. We worked in the stable, participated in the CCC symposium, and visited industrial partners together, these are unforgettable experiences. "Hi Gert" is usually enough to start our conversation. You are a very kind professor, who is always smiling. Thank you for your great support, Gert!

Prof. dr. W.J.J. Gerrits, dear Walter, you have guided me through my PhD project and gave me lots of support regarding the *in vivo* part of my thesis. We had many pleasant conversations during the CCC meetings. You brought peace, perspective and professionalism and proposed valuable suggestions when necessary. You brought me interesting knowledge and taught me how to handle questions beyond my background of pharmacology and immunology. Thank you very much for your kind help, Walter!

Dr. S. Braber, dear Saskia, elegant Saskia, you are so nice and professional! Thanks to your immense patience and valuable advice, my academic writing no longer looks like a “fairy tale”. Your talent and ambitions taught me how to conduct research in a proper way and formulate appropriate plans. We worked together in the laboratory and in the stable for a long time, and we had so many pleasant and friendly conversations. We can talk and share everything at any time via app, including experiments, life, photos, food, family, happiness, sadness, etc., These experiences allowed me to stay positive, work independently and this contributed to the raising awareness of the promising effects of oligosaccharides. Without your enthusiasm and patience, this thesis would never be what it is today. Thank you for your attentive help, Saskia!

Committee

Prof. dr. A.D. Kraneveld, Prof. dr. H.J. Wichers, Prof. dr. N.M. van Sorge, Prof. dr. R. Gosens, and Dr. N. Stockhofe-Zurwieden, thank you very much for carefully reading and assessing my thesis.

Project research group

Prof. dr. J.P.M. van Putten, dear Jos, it was my pleasure to be trained in the field of microbiology in your laboratory, which was a totally new area for me. Your opinion and suggestions have impressed me and supported my thesis outstandingly. Thank you very much, Jos!

Dr. M.S. Gilbert, dear Myrthe, working with you was a surprising consequence. I have never thought that I would participate in calf trials before. But here, your professional skills in the field of zoology gave me a wealth of fresh knowledge and opened new horizons. You always gave me quick feedback and provided appropriate support regarding the *in vivo* part of my project. Thank you for your kind support!

Dr. W.W.J. Unger, dear Wendy, the collaboration with you gave me the opportunity to connect our preclinical data to the clinic. I am so happy that I could include *in vitro*, *in vivo* and “clinical” (human) data in my thesis to emphasize the importance of translation research. The experience in your laboratory and the discussions together with you inspired me. Thank you for your kind help, and I am very happy that our joint project is continuing!

Paranymphs

Dear Wang Lei and Zheng Yuanpeng, thank you for your enthusiasm and positive energy, which made my stay in the Netherlands interesting. I am proud that you are my friends, and I enjoyed our conversations. Lei, thank you for your help in the lab and I am grateful for those warm memories that we made during traveling. I sincerely wish you both a successful thesis and dissertation!

Colleagues

Gratitude to my lovely UIPS colleagues, we can always meet each other at the corner of our department, and we have worked and talked happily together. UIPS colleagues always gave enthusiastic help immediately, which made me feel very welcome and happy. Dear Aletta, Anne Metje, Amer, Amir, Adel, Astrid, Bart, Betty, Charlotte, Chantal, Emine, Frank, Gemma, Hamed, Ingrid, Jelle, Joao, Jose,

Karin, Koen, Katja, Linette, Lidija, Mara, Monika, Mehrdad, Mirelle, Marit, Negisa, Naika, Paul, Pieter, Paula, Roos, Sandra, Suzan, Suzanna, Thea, Veronica, thank you very much for your help, explanation and listening ear.

Dear Johan Garssen, the head of Pharmacology, thank you for leading and supporting our kind and warm pharmacology family! This allowed me to conduct my project effectively.

Dear Paul Henricks, thank you for organizing those interesting PhD programs and thank you for improving my Dutch summary!

Dear Soheil Varasteh, thank you for your help during my animal experiment! It always feels comfortable to work and talk with you.

Dear Lisa Slimmen and Hongzhen Zhu, I am very happy to collaborate with you at Erasmus University Medical Center. Your excellent scientific skills and ideas have benefited me a lot!

Colleagues from China

在国外的五年岁月里，感谢陪伴着一起成长的朋友，当中有些已经回去了家乡，有些还跟我一样身在他国。但我想，也正因为彼此的陪伴才让这些国外的时光变得温暖而柔和。陈婧，李梦，连浦峤，刘德光，田时祎，王振国，王蕾，杨逸，赵小莉，赵玉泷，郑远鹏，曾亚龙，谢谢你们！有你们一起，纵使疫情风雨，科研枯燥，也有一份闲情逸致和生活气息。

“海内存知己，天涯若比邻“，愿你们都可以一切顺心！

Family

最后，我要感谢我的祖国，伟大的中国，感谢祖国给予我出国的机会！我更要感谢我的父母，我的家人，我的女友，谢谢你们的陪伴与支持！在家人面前，文字总是过于苍白，但家人的存在便如黑夜之光，冬日之火，这火光照亮的地方，便是家的方向！

写到最后，我想起了那首《再别康桥》，以此结尾吧！
寻梦？撑一支长篙/向青草更青处漫溯/满载一船星辉/在星辉斑斓里放歌。
但我不能放歌/悄悄是别离的笙箫/夏虫也为我沉默/沉默是今晚的“康桥”！

Curriculum Vitae

Curriculum Vitae

BIRTH — 1991
Yang Cai was born on July 05th 1991 in Jiangsu, China

2013 — BACHELOR OF ENGINEERING
He graduated from Pharmaceutical Engineering, Faculty of Pharmacy, Jiangsu University

2016 — MASTER OF MEDICINE
He researched neuroinflammation and pain and graduated from Department of Pharmacology, Faculty of Basic Medicine, Nanjing Medical University

- Intern at Merck & Co
- Project manager at Shanghai-biotech Company

2017 — PHD CANDIDATE
As a PhD candidate at UIPS, he researched the benefits of non-digestible carbohydrates in the intervention of lung infection and inflammation

2021 — PHD
He got his PhD at Utrecht University, under the supervision of Dr. S. Braber, Prof. Dr. W.J.J. Gerrits, and Prof. Dr. G. Folkerts

- Present: Research on two joint projects

X

List of publications

This thesis

Yang Cai, Soheil Varasteh, Jos P.M. van Putten, Gert Folkerts, and Saskia Braber. *Mannheimia haemolytica and lipopolysaccharide induce airway epithelial inflammatory responses in an extensively developed ex vivo calf model.* Scientific Reports 10, 13042 (2020). [Chapter 2]

Yang Cai, Jelle Folkerts, Gert Folkerts, Marcus Maurer, and Saskia Braber. *Microbiota-dependent and -independent effects of dietary fibre on human health.* British Journal of Pharmacology 177:1363-1381 (2020). [Chapter 8]

Yang Cai, Myrthe S. Gilbert, Walter J.J. Gerrits, Gert Folkerts, and Saskia Braber. *Galacto-oligosaccharides alleviate lung inflammation by inhibiting NLRP3 inflammasome activation in vivo and in vitro.* Journal of Advanced Research (Revision submitted). [Chapter 4]

Yang Cai, Jos P.M. van Putten, Myrthe S. Gilbert, Walter J.J. Gerrits, Gert Folkerts, and Saskia Braber. *Galacto-oligosaccharides as an anti-bacterial and anti-invasive agent in lung infections.* Biomaterials (Revision). [Chapter 5]

Yang Cai, Myrthe S. Gilbert, Walter J.J. Gerrits, Gert Folkerts, and Saskia Braber. *Anti-inflammatory properties of fructo-oligosaccharides in a calf lung infection model and in Mannheimia haemolytica-infected airway epithelial cells.* Submitted. [Chapter 6]

Yang Cai, Lisa Slimmen, Hongzhen Zhu, Gert Folkerts, Saskia Braber, and Wendy W.J. Unger. *Bacteriostatic Effect of Fructo-oligosaccharides and Bactericidal Effect of Galacto-oligosaccharides on Mycoplasma Pneumoniae in vitro.* Ready for submission. [Chapter 7]

Yang Cai, Myrthe S. Gilbert, Walter J.J. Gerrits, Gert Folkerts, and Saskia Braber. *Naturally Occurring Respiratory Infection Model in Calves: Development of Neutrophil-driven Inflammation.* Preparation. [Chapter 3]

Myrthe S. Gilbert, Yang Cai, Saskia Braber, Gert Folkerts, and Walter J.J. Gerrits. *Effects of oligosaccharides supplementation on lung health and performance of veal calves.* Preparation.

Other publications

Myrthe S. Gilbert, Yang Cai, Saskia Braber, Gert Folkerts, and Walter J.J. Gerrits. *Effects of microbiota transplantation with or without galacto-oligosaccharides supplementation on inflammation, lung health and performance of veal calves.* Preparation.

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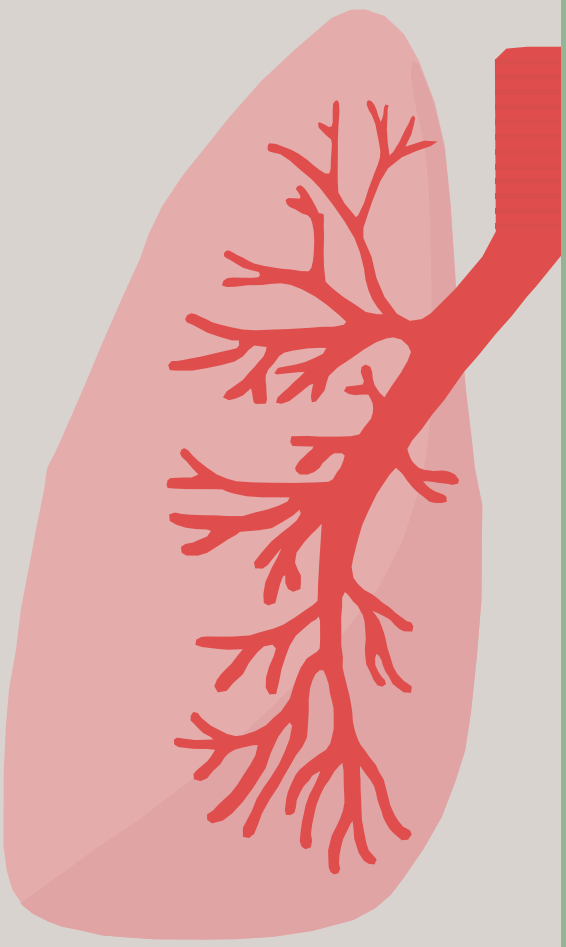
Negisa Seyed Toutounchi, Saskia Braber, Astrid Hogenkamp, Soheil Varasteh, Yang Cai, Tjalling Wehkamp, Sebastian Tims, Thea Leusink-Muis, Ingrid van Ark, Selma Wiertsema, Bernd Stahl, Aletta D. Kraneveld, Johan Garssen, Gert Folkerts, and Belinda van't Land. *Human milk oligosaccharide 3'-GL improves Influenza-specific vaccination responsiveness and immunity after deoxynivalenol exposure in preclinical models.* Nutrients (2021).

----------The End----------



ISBN 978-94-6416-770-2

Oligosaccharides to suppress respiratory infections: A translational approach

YANG CAI