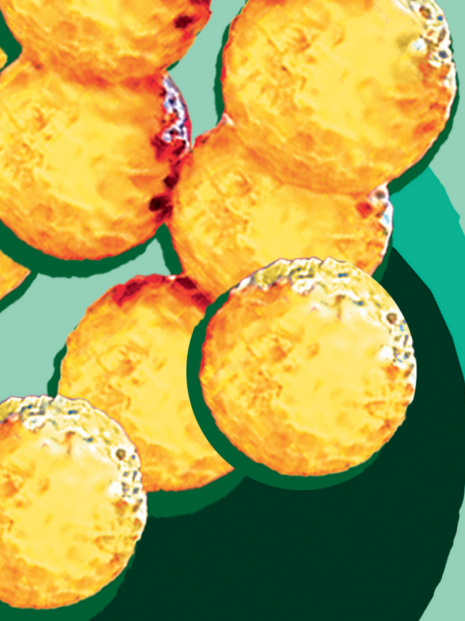

Anti-Pathogenic Properties of Non-Digestible Oligosaccharides

The fight against bacterial pathogens and toxins

Mostafa Asadpoor (Adel)

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Het gevecht tegen pathogene bacteriën en toxinen

(met een samenvatting in het Nederlands)

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Contents

Chapter 1

Introduction

1- Bacterial infections

A bacterium is a single but complex cell and performs multiple critical functions in maintaining the human body homeostasis. For example, vitamins K and B are mostly produced by human gut bacteria and these bacteria are the principal metabolizers of sterols and bile acids in the body (1). Only a very small percentage of the world's bacteria can cause infection and disease in humans (less than 1% of all bacterium types), for example bacteria like *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli*, and *Clostridium difficile* (2,3). In spite of successful prevention and control efforts within the last decades, infectious diseases are still an important worldwide public health problem, causing more than 13 million deaths annually (4). Bacterial pathogens can cause an infectious disease, when they are capable to reach their target tissue, proliferate and overcome host barriers and evade the host immune response (5). Several host factors are important in predicting whether illness will develop once a bacterial pathogen is transmitted. Genetic make-up, immunological and nutritional state, age, and length of exposure to the organism are all factors to consider (2, 3, 6).

2- Antibiotics

The discovery and development of antibiotics is one of the most important medical developments of the twentieth century (7) and played a key role in preventing millions of deaths due to bacterial infections (8). Antibiotics are split into two types based on their working mechanism: bactericidal antibiotics that kill bacteria and bacteriostatic antibiotics that inhibit bacteria from reproducing or growing. The bactericidal mode of action of antibiotics can include inhibiting cell wall synthesis, inhibiting bacterial enzymes or protein translation. Various categories of antibiotics belong to this class, including beta-lactams, fluoroquinolones, and glycopeptides. Aminoglycosidic antibiotics are often considered bactericidal, however, they might be bacteriostatic to some organisms (9). Bacteriostatic antibiotics limit the growth of bacteria by interfering with bacterial DNA replication, protein production, or bacterial cellular metabolism. Tetracyclines, sulfonamides, and macrolides are the most famous categories belonging to this class of antibiotics. Furthermore, it should be considered that there is not always a precise distinction between bacteriostatic and bactericidal antibiotics. High concentrations of some bacteriostatic agents could also be bactericidal (9).

3- Bacterial resistance

Nowadays the mounting spread of bacterial resistance becomes a serious worldwide challenge which threatens the huge successes of antibiotic therapies (8). Antibiotic resistance is a capability that bacteria develop to defeat the drugs designed to kill them. There are different mechanisms of antimicrobial resistance in bacteria such as, biofilm formation, alteration of bacterial proteins that are antimicrobial targets, enzymatic

degradation of antibacterial medicines, and changes in membrane permeability to antibacterial agents (10). Importantly, across all microbial and chronic illnesses, biofilm formation is the most prevalent bacterial resistance mechanism (> 65%) (11, 12). A biofilm is a thick layer of bacteria within a matrix of extracellular polymeric substances that have aggregated to form a colony and prevents the penetration of antimicrobial agents (13). Current antibacterial tactics confront significant challenges, including a paucity of effective medications and a scarcity of novel antibiotics in the clinical pipeline, which will necessitate the creation of novel treatment approaches and alternative antibiotics.

4- Toxins

Toxins are formed by a broad range of pathogens attacking host cells and playing key roles in bacterial pathogenesis. They are the most important virulence factors of bacteria and are often the cause of the corresponding bacterial infection (14). Bacterial toxins can be single proteins or oligomeric protein complexes (15) and can be classified as either exotoxins or endotoxins. Exotoxins are generated and actively released into the surrounding environment (e.g. cholera toxin). Endotoxins (e.g. lipopolysaccharides) are present in almost all gram-negative bacteria and strongly activate the immune system. Endotoxins are not released until the bacterium disintegrates by e.g. antimicrobial agents. Bacterial toxins target different receptors and act via different mechanisms of action. But beyond all these differences, the release of toxins often leads to disruption of metabolic pathways of the eukaryote host, including protein synthesis disruption, cell membrane damage, or activation of the host immune system (16, 17).

5- Intestinal epithelial barrier

The gut barrier is vital for maintaining intestinal and systemic homeostasis, absorbing fluids (and nutrients), separating the external and internal environment and protect the body from microorganisms and harmful antigens (18). The intestinal epithelium is a single layer of cells that forms the luminal surface of the intestine in the gastrointestinal tract. The epithelial layer is the first line of host defense against infectious agents in the human intestine. Cell-cell junctional complexes between the intestinal epithelial cells (IECs) form a tight barrier that strictly regulates paracellular permeability and are pivotal for the integrity of the gut barrier (19). In addition, IECs are important players in preserving the delicate balance between gut tolerance and inflammation. IECs express various cytokine/chemokine receptors and also have the ability to release cytokines and chemokines, which affect and mediate the infiltration and activation of immune cells (20). Disrupted barrier integrity is directly linked to increased paracellular and transcellular intestinal permeability, an indication of increased invasion of pathogens into the systemic circulation with subsequent systemic inflammation and initiation of several (inflammatory) disorders (21). Confirmed by all above mentioned, the preservation of a homeostatic intestinal epithelial barrier is highly imperative.

6- Non-digestible oligosaccharides (NDOs)

In recent years, there has been a growing interest in functional foods/nutraceuticals that have the capacity to enhance human health. Dietary carbohydrates, particularly NDOs, have been introduced as an important class of industrial functional food ingredients. NDOs are known to selectively enhance the growth and/or activity of beneficial bacteria in the intestine, specifically Bifidobacteria and Lactobacilli and, thus, recognized as prebiotics (22). To reach and be effective in the gastrointestinal tract, NDOs are resistant to hydrolysis by digestive enzymes in the upper part of the intestines. There is mounting evidence that health-promoting activities of NDOs are not restricted to shape the intestinal microbiota and the microbiota-related immune responses, but also include microbiota-independent effects on immune and epithelial cells (23–25). Furthermore, supplementing NDOs to newborns has been shown to reduce the development of disorders, such as allergies (26). However, NDOs can also induce therapeutic effects in different inflammatory diseases later in life, including colitis, lung emphysema, cancer and HIV (27). Recently, there is growing interest in the anti-pathogenic properties of NDOs to prevent or/and treat various kinds of infections, including respiratory and gastrointestinal infections (28, 29).

NDOs, characterized by various structural features, can block the pathogenicity of pathogenic bacteria via various mechanisms of action. It has been reported that NDOs can directly inhibit the proliferation of pathogenic bacteria (30, 31). NDOs are also capable to act as anti-adhesives to selectively avert adhesion of a specific pathogen species to human cells. The anti-adhesive effects of NDOs are thought to be due to their structural resemblance to oligosaccharide patterns seen on host cell surface proteins (32). Furthermore, NDOs have anti-biofilm capabilities against diverse pathogenic microbes. In addition, recent investigations highlight the anti-inflammatory capabilities of NDOs *in vitro* as well as *in vivo* (33–35).

7- Aims and outlines of the thesis

NDOs have prebiotic properties by stimulating the growth of beneficial bacteria in the intestine. However, the capabilities of NDOs may go beyond, since there are indications that NDOs can directly interact with pathogenic bacteria. To date, NDOs exhibited various pathogen reduction capabilities, and thus may represent potential therapeutic candidates against infections. Inspired by those findings, the aim of this thesis is to investigate the unknown strategies of NDOs in defense against several pathogenic bacteria, including gram-negative and gram-positive bacteria, which are targeting the human intestine and causing (early life) infections. Moreover, this thesis investigates whether certain NDOs can improve the effectiveness of conventional antibiotics. Several in vitro microbiological and pharmacological investigations have been carried out for this purpose.

In the following, the content of each chapter is described:

In **Chapter 2** *in vitro* anti-pathogenic capabilities of several NDOs and related pathways are discussed in detail. A framework is developed that categorizes all anti-pathogenic actions and elucidates the structural requirements for a NDO to exhibit one of these effects.

In **Chapter 3** the effects of NDOs (alginate-oligosaccharides, chitosan-oligosaccharides, galacto-oligosaccharides and fructo-oligosaccharides) on *Escherichia coli* growth, adhesion and on *Escherichia coli*-induced inflammatory response (IL-8 release) of HT-29 intestinal epithelial cells were determined *in vitro* in the presence or absence of an antibiotic (ampicillin).

In **Chapter 4** the antimicrobial potential of alginate oligosaccharides and chitosan oligosaccharides as alternative for, or in combination with, several antibiotics (ampicillin, trimethoprim, ciprofloxacin, tetracycline, clindamycin) against *Staphylococcus aureus* and group *B streptococcus* was investigated.

Chapter 5 is a comprehensive overview that focuses on the strategies of enteropathogenic bacteria (EPB) and related enterotoxins to impair host cell immunity, discusses the anti-pathogenic properties of NDOs and short-chain fatty acids (SCFAs) on EPB functions, and offers insight into the potential use of NDOs and SCFAs as effective agents to combat enterotoxins.

In **Chapter 6** the hypothesis is investigated whether alginate- and chitosan-oligosaccharides possess antipathogenic and barrier-protective properties against *Clostridium difficile* bacteria and TcdA toxin, respectively.

In **Chapter 7** we tried to find an effective inhibitor of Shiga toxin or Shiga-like toxin (Stx) (produced by *Shigella* species or *Escherichia coli*) by comparing three classes of carbohydrate-based inhibitors: glycodendrimers, glycopolymers, and oligosaccharides.

Chapter 8 summarizes the most relevant findings of all studies and provides suggestions for future research activities.

References

1. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, et al. Gut microbiota functions: metabolism of nutrients and other food components. Eur J Nutr 2018;57(1):1-24. https://doi.org/10.1007/s00394-017-1445-8.
2. Sanchez E. Bacterial Infections: Overview. Ref Modul Biomed Sci 2017. https://doi.org/10.1016/b978-0-12-801238-3.03000-2.
3. Revelas A. Healthcare - associated infections: A public health problem. Niger Med J 2012;53(2):59. https://doi.org/10.4103/0300-1652.103543.
4. Cohen ML. Changing patterns of infectious disease. Insight Rev Artic 2000;406(6797):762-767. https://doi.org/10.1038/35021206.
5. Nina Parker, Mark Schneegurt, Anh-Hue Thi Tu, Philip Lister BMF. Microbiology. OpenStax; 2016. http://keep.hcmiu.edu.vn:8080/handle/123456789/3529.
6. Stadler M, Dersch P. How to Overcome the Antibiotic Crisis. 2017;398.
7. Santesmases MJ, Gradmann C. Circulation of antibiotics: An introduction. Dynamis 2011;31(2):293-303. https://doi.org/10.4321/s0211-95362011000200002.
8. Friedman ND, Temkin E, Carmeli Y. The negative impact of antibiotic resistance. Clin Microbiol Infect 2016;22:416–22. https://doi.org/10.1016/j.cmi.2015.12.002.
9. Alekshun MN, Levy SB. Molecular Mechanisms of Antibacterial Multidrug Resistance. Cell 2007;128(6):1037–50. https://doi.org/10.1016/j.cell.2007.03.004.
10. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJV. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 2015;13:42–51. https://doi.org/10.1038/nrmicro3380.
11. Venkatesan N, Perumal G, Doble M. Bacterial resistance in biofilm-associated bacteria. Future Microbiol 2015;10(11):1743–50. https://doi.org/10.2217/fmb.15.69.
12. Jamal M, Ahmad W, Andleeb S, Jalil F, Imran M, Nawaz MA, et al. Bacterial biofilm and associated infections. J Chinese Med Assoc 2018;81:7–11. https://doi.org/10.1016/j.jcma.2017.07.012.
13. Flemming H-C, Wingender J. The biofilm matrix. Nat Rev Microbiol 2010;8:623–33. https://doi.org/10.1038/nrmicro2415.
14. Sarkar P, Issac PK, Raju S V., Elumalai P, Arshad A, Arockiaraj J. Pathogenic bacterial toxins and virulence influences in cultivable fish. Aquac Res 2021;52:2361–76. https://doi.org/10.1111/are.15089.
15. Birkhäuser Verlag. Molecular, Clinical and Environmental Toxicology. Clin Toxicol 2010;2. https://link.springer.com/content/pdf/10.1007/978-3-7643-8338-1.pdf.
16. Asadpoor M, Ithakisiou G.N PAJH, Pieters R, Folkerts G, Braber and S. Non-Digestible Oligosaccharides and Short Chain Fatty Acids as Therapeutic Targets against Enterotoxin-Producing Bacteria and Their Toxins. Toxins (Basel) 2021;13:175. https://doi.org/10.3390/toxins13030175.
17. Cavaillon JM. Exotoxins and endotoxins: Inducers of inflammatory cytokines. Toxicon 2018;149:45–53. https://doi.org/10.1016/j.toxicon.2017.10.016.
18. Schoultz I, Keita Å V. The Intestinal Barrier and Current Techniques for the Assessment of Gut Permeability. Cells 2020;9:1–30. https://doi.org/10.3390/cells9081909.
19. Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. J Nutr 2011;141:769–76. https://doi.org/10.3945/jn.110.135657.
20. Kulkarni N, Pathak M, Lal G. Role of chemokine receptors and intestinal epithelial cells in the mucosal inflammation and tolerance. J Leukoc Biol 2017;101:377–94. https://doi.org/10.1189/jlb.1ru0716-327r.
21. Hollander D, Kaunitz JD. The "Leaky Gut": Tight Junctions but Loose Associations? Dig Dis Sci 2020;65:1277–87. https://doi.org/10.1007/s10620-019-05777-2.
22. Mussatto SI, Mancilha IM. Non-digestible oligosaccharides: A review. Carbohydr Polym 2007;68:587–97. https://doi.org/10.1016/j.carbpol.2006.12.011.

23. Akbari P, Fink-Gremmels J, Willems RHAM, Difilippo E, Schols HA, Schoterman MHC, et al. Characterizing microbiota-independent effects of oligosaccharides on intestinal epithelial cells: insight into the role of structure and size: Structure–activity relationships of non-digestible oligosaccharides. Eur J Nutr 2017;56:1919–30. https://doi.org/10.1007/s00394-016-1234-9.
24. Cai Y, Folkerts G, Maurer M. Microbiota - dependent and - independent effects of dietary fibre on human health 2020;177(6):1363–81. https://doi.org/10.1111/bph.14871.
25. Wongkrasant P, Pongkorpsakol P, Ariyadamrongkwan J, Meesomboon R, Satitsri S, Pichyangkura R, et al. A prebiotic fructo-oligosaccharide promotes tight junction assembly in intestinal epithelial cells via an AMPK-dependent pathway. Biomed Pharmacother 2020;129:110415. https://doi.org/10.1016/j.biopha.2020.110415.
26. Schouten B, van Esch BCAM, Kormelink TG, Moro GE, Arslanoglu S, Boehm G, et al. Non-digestible oligosaccharides reduce immunoglobulin free light-chain concentrations in infants at risk for allergy. Pediatr Allergy Immunol 2011;22:537–42. https://doi.org/10.1111/j.1399-3038.2010.01132.x.
27. Algieri F, Rodríguez-Nogales A, Garrido-Mesa N, Vezza T, Garrido-Mesa J, Utrilla MP, et al. Intestinal anti-inflammatory effects of oligosaccharides derived from lactulose in the trinitrobenzenesulfonic acid model of rat colitis. J Agric Food Chem 2014;62:4285–97. https://doi.org/10.1021/jf500678p.
28. Okeke IN, Aboderin OA, Byarugaba DK, Ojo KK, Opintan JA. Growing problem of multidrug-resistant enteric pathogens in Africa. Emerg Infect Dis 2007;13:1640–6. https://doi.org/10.3201/eid1311.070674.
29. Humphries RM, Schuetz AN. Antimicrobial susceptibility testing of bacteria that cause gastroenteritis. Clin Lab Med 2015;35:313–31. https://doi.org/10.1016/j.cll.2015.02.005.
30. Asadpoor M, Peeters C, Henricks PAJ, Varasteh S, Pieters RJ, Folkerts G, et al. Anti-pathogenic functions of non-digestible oligosaccharides in vitro. Nutrients 2020;12:1789. https://doi.org/10.3390/nu12061789.
31. Asadpoor M, Ithakisiou GN, van Putten JPM, Pieters RJ, Folkerts G, Braber S. Antimicrobial Activities of Alginate and Chitosan Oligosaccharides Against Staphylococcus aureus and Group B *Streptococcus*. Front Microbiol 2021;12:700605. https://doi.org/10.3389/fmicb.2021.700605.
32. Bernardi A, Jiménez-barbero J, Casnati A, Castro C De. Multivalent glycoconjugates as anti-pathogenic agents † 2015;42:4709–27. https://doi.org/10.1039/c2cs35408j.Multivalent.
33. Zhang W, He-Yang J, Tu W, Zhou X. Sialylated human milk oligosaccharides prevent intestinal inflammation by inhibiting toll like receptor 4/NLRP3 inflammasome pathway in necrotizing enterocolitis rats. Nutr Metab 2021;18:1–11. https://doi.org/10.1186/s12986-020-00534-z.
34. Cai Y, Gilbert MS, Gerrits WJJ, Folkerts G, Braber S. Galacto-oligosaccharides alleviate lung inflammation by inhibiting NLRP3 inflammasome activation in vivo and in vitro. J Adv Res 2021;39:305-318. https://doi.org/10.1016/j.jare.2021.10.013.
35. Newburg DS, Ko JS, Leone S, Nanthakumar NN. Human milk oligosaccharides and synthetic galactosyloligosaccharides contain 3'-, 4-, and 6'-galactosyllactose and attenuate inflammation in human T84, NCM-460, and H4 cells and intestinal tissue ex vivo1,2. J Nutr 2016;146:358–67. https://doi.org/10.3945/jn.115.220749.

Chapter 2

Anti-Pathogenic Functions of Non-Digestible Oligosaccharides *In Vitro*

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Abstract

Non-digestible oligosaccharides (NDOs), complex carbohydrates that resist hydrolysis by salivary and intestinal digestive enzymes, fulfill a diversity of important biological roles. A lot of NDOs are known for their prebiotic properties by stimulating beneficial bacteria in the intestinal microbiota. Human milk oligosaccharides (HMOs) represent the first prebiotics that humans encounter in life. Inspired by these HMO structures, chemically-produced NDO structures (e.g., galacto-oligosaccharides and chito-oligosaccharides) have been recognized as valuable food additives and exert promising health effects. Besides their apparent ability to stimulate beneficial microbial species, oligosaccharides have shown to be important inhibitors of the development of pathogenic infections. Depending on the type and structural characteristics, oligosaccharides can exert a number of anti-pathogenic effects. The most described effect is their ability to act as a decoy receptor, thereby inhibiting adhesion of pathogens. Other ways of pathogenic inhibition, such as interference with pathogenic cell membrane and biofilm integrity and DNA transcription, are less investigated, but could be equally impactful. In this review, a comprehensive overview of *in vitro* anti-pathogenic properties of different NDOs and associated pathways are discussed. A framework is created categorizing all anti-pathogenic effects and providing insight into structural necessities for an oligosaccharide to exert one of these effects

Keywords: non-digestible oligosaccharides; bacteria; bacterial growth; biofilm; adhesion; surface charge; chemical structure; HMOs; *in vitro*

1. Introduction

In recent years, there has been a growing interest in functional foods/nutraceuticals that have the ability to enhance human health, resulting in one of the leading trends in today's food industry. Dietary carbohydrates, especially non-digestible oligosaccharides (NDOs), have been introduced as functional food ingredients. NDOs are known to selectively promote the growth and/or activity of beneficial bacteria in the gut, especially Lactobacilli and Bifidobacteria and, therefore, recognized as prebiotics (1). To reach and be effective in the large intestine, NDOs are resistant to hydrolysis by intestinal digestive enzymes in the upper part of the intestines.

There is a great body of evidence that health-promoting effects of NDOs are not limited to shaping the intestinal microbiota and the microbiota-associated immune responses, but also include microbiota-independent effects on epithelial and immune cells (2). It has been described that supplementation of NDOs to the diet in early life can decrease the development of diseases, such as allergies (3). However, NDOs can also induce therapeutic effects in different inflammatory diseases later in life, including colitis, lung emphysema, cancer and HIV (4). Recently, there has been particular scientific interest in the anti-pathogenic properties of NDOs for treatment (or prevention) of several kinds of infections, including gastrointestinal and respiratory infections (5,6). Especially, antibiotic-resistant bacteria pose a great threat to human health and are associated with a major cause of morbidity and mortality worldwide (7). Adhesion to host proteins (saccharides patterns) and biofilm development are thought to be two important pathogenic mechanisms. Bacterial biofilm formation is associated with a wide range of infections and reduces pathogenic susceptibility to antibiotic treatment. The multicellular nature of biofilms prevents the penetration of antimicrobial agents. Aggravation of antibiotic resistance among pathogenic species has urged development of alternative treatments for infections (8).

The microbiota protect against infections by promoting beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus*, by inhibiting pathogenic bacteria or by orchestrating appropriate immune responses, therefore NDOs can play an important role in treating infections (9). This topic has been extensively reviewed in recent publications (10–12).

NDOs can also act as anti-adhesives to selectively prevent adhesion of certain pathogen species to human cells and to mucin. For their adhesion-inhibiting properties, NDOs rely on structural similarity with oligosaccharide patterns presented on proteins on the host cell surface (13). These patterns are essential for fimbria/pili-mediated pathogenic adhesion, allowing for anti-pathogenic capability termed receptor-mimicry (14–16). In addition, it has been reported that NDOs possess anti-biofilm activity against different pathogenic microbes. NDOs can inhibit the development of pathogenic infection of the intestine before pathogen adhesion (14,17,18) or during one of the initial stages of biofilm formation (19–21) through direct interaction with pathogens.

Human milk contains a large amount of structurally diverse oligosaccharides, termed human milk oligosaccharides (HMOs), which represent the first prebiotics that humans encounter in life. Each structurally defined HMO might have a distinct functionality related to their anti-pathogenic properties. Inspired by the prebiotic and anti-pathogenic potential of HMOs, similar oligosaccharide structures were tested for their anti-pathogenic capability (14). Some of the oligosaccharides produced are based on monosaccharides also present in HMOs, such as galactose (Gal) in galacto-oligosaccharides (GOS) and N-acetylglucosamine (GlcNAc) in chito-oligosaccharides (COS). Other commercial oligosaccharides with anti-pathogenic potential include mannan-oligosaccharides (MOS), alginate oligosaccharide (AOS), pectic oligosaccharides (POS) and fructo-oligosaccharides (FOS). These commercial NDOs can be obtained by direct extraction from natural sources or produced via enzymatic or chemical synthesis from saccharides (22). There is a high structural diversity amongst these NDOs and depending on their key characteristics, such as monosaccharide components, charge, degree of polymerization (DP) and degree of acetylation (DA), these oligosaccharides elicit anti-pathogenic effects in a variety of ways.

In this review, an extensive overview of the anti-pathogenic effects of different NDOs and their postulated mechanisms are addressed. Herein, the focus lies on direct interaction of oligosaccharides with pathogens or components of the biofilm. Since the NDO-induced effects on the microbiota and microbiota-generated metabolites cannot be neglected in vivo, only *in vitro* studies are included. A framework is created categorizing all anti-pathogenic effects of relevant NDOs and providing insight into the structural requirements for an oligosaccharide to exert one of these effects.

2. Human Milk Oligosaccharides

2.1. Structure

HMOs are soluble complex and diverse sugars containing Gal, Glc, fucose (Fuc), N–acetylglucosamine (GlcNAc), or sialic acid (Neu5Ac) monosaccharides. In the mammary glands, the HMOs are biosynthesized with the formation of a lactose core from Glc and Gal catalyzed by β–galactotransferase in the presence of α–lactalbumin. Galactose can be elongated enzymatically by β1–4 linkage to N-acetyllactosamine or by β1–3 linkage to lacto-N-biose. The core HMO structure can be further elongated by the addition of N-acetyllactosamine and lacto-n-biose units by β1–6 and β1–3 linkages; Fuc connected with α1–3, α1–2, or α1–4 linkages; and/or sialic acid residues attached by α2–6 or α2–3 linkages at the terminal positions (Figure 1) (23). Human milk contains three major HMO types: neutral (Fucosylated) HMOs (e.g., 2-fucosyllactose (2-FL)), neutral N-containing HMOs (lacto-N-tetraose (LNT)) and acidic (sialylated) HMOs (e.g., 3-sialyllactose (3-SL)) (24). Nutrients 2020, 12, 1789 3 of 31 β–galactotransferase in the presence of α–lactalbumin. Galactose can be elongated enzymatically by β1–4 linkage to N-acetyllactosamine or by β1–3 linkage to lacto-N-biose. The core HMO structure can be further elongated by the addition of N-acetyllactosamine and lacto-N-biose units by β1–6 and β1–3 linkages; Fuc

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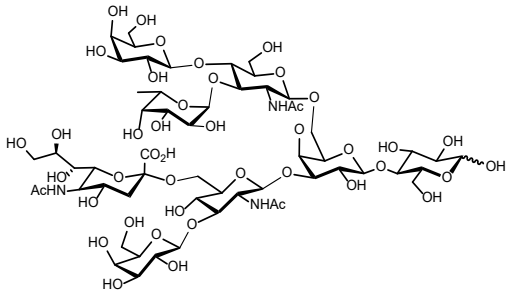

Figure 1. Exemplary structure of an HMO.

2.2. Anti-Pathogenic Functionalities HMO Mixtures

HMOs have been shown to interact with pathogenic bacteria in a variety of ways (Table 1). Depending on their structural characteristics, HMOs may interact with adhesion factors on the pathogenic surface, or penetrate and interact with elements of the pathogenic biofilm, inhibiting microbial adhesion and biofilm growth (25,26). When pathogens bind specific HMOs that resemble saccharide structures on the epithelial cell surface, their capacity to adhere to epithelial cells is inhibited.

2.2.1. Anti-Adhesion HMO Mixtures

HMO structures binding to pathogenic fimbriae by resembling patterns on epithelial cell surface receptors are called decoy receptors (25).The ability of HMOs to influence pathogenic adhesion is influenced by number of variables, such as the percentage of fucosylated or acidic oligosaccharides in the mixture (the fucosylated and sialylated fraction, respectively), oligosaccharide weight or the type of pathogen.

Neutral HMO Fraction—Fucosylated and Non-Fucosylated

Neutral HMO fractions are known to inhibit adhesion of pathogens to epithelial cells. As a whole, the neutral fraction of HMOs inhibits an *Escherichia coli* strain, which is specifically P-fimbriated. i.e., galabiose (or galactose) specific, indicating a mechanism of receptor-mimicry (27). After separation of a neutral oligosaccharide mixture into a high-and low-molecular weight fraction (HMWF and LMWF, respectively), the two fractions showed varying adhesion-inhibiting potential depending on the pathogen strain. The HMWF showed inhibition of *Vibrio cholerae* adhesion, whereas the LMWF inhibited *Salmonella fyris* (17), which contrasts the finding that the LMWF more potently inhibits *Vibrio cholerae* adhesion (28). Larger HMOs appear to have an advantage over smaller HMOs in inhibiting the adhesion of pulmonary infectious strains, such as *Haemophilus influenzae*, however, the effect is also dependent on the composition of the HMO mixture (29). One

factor affecting pathogen adhesion to epithelial cells is the fucosylation status of the HMO mixture. Oligosaccharides are fucosylated by FUT2 (α1,2–fucose) or FUT3 (α1,4–fucose) (25). In general, α1,2–fucose patterns on epithelial cell exterior have been shown to protect epithelial cells from pathogenic infection by facilitating colonization of a layer of probiotic microbes (30,31). In addition, locally secreted glycan molecules may inhibit pathogenic colonization through a decoy-receptor mechanism, although this mechanism has not been fully elucidated (31,32).

In accordance with anti-colonization functionalities exerted by native fucosyl-containing elements, α1,2–fucosylated HMOs have also shown to exert anti-adhesion effects. A large part of the neutral oligosaccharide fraction is made up of fucosylated oligosaccharides, which is usually present at the reducing end of the oligosaccharide sequence (33). Small fucosylated HMOs inhibit enteropathogenic *Escherichia coli* (EPEC) adhesion, when added to a HEP-2 monolayer along with EPEC (34), while fucosylated oligosaccharides show superior adhesion inhibition of *Neisseria meningitidis* to salivary agglutinin (35). Although these results point in the direction of receptor mimicry functionalities of the neutral fraction, the anti-pathogenic effect cannot be tracked to a specific saccharide structure as the molecular diversity in these mixtures is very high (36). Testing of isolated HMOs gives a better indication of the inhibitory capacities compared to testing of HMOs in mixture, this is discussed in the Isolated HMO structures section.

Acidic HMO Fraction

The acidic fraction of HMOs consists of sialylated oligosaccharides, which are negatively charged at homeostatic pH. Sialylated HMOs are produced by the action of sialyltransferase enzymes resulting in α2,3 and α2,6–sialylated oligosaccharides (25). Due to the charge residing on the sialylated oligosaccharides, and their consequent interaction with oppositely charged elements on the epithelial cell exterior, their adhesion-inhibiting effect is less dependent on pathogen type compared to the neutral fraction (17). Similar to neutral HMO fractions, the acidic HMO fraction shows inhibitory potential towards pathogenic species expressing specific fimbrial types, such as P and CFA fimbriae-expressing Escherichia coli. The lack of inhibition of HMO's for P-fimbriated *Escherichia coli* is clarified by the lack of affinity of the P-fimbrial lectin for sialylated oligosaccharides instead of the Galα1,4 Gal (galabiose) termini on the cell surface, which are involved in recognition and adhesion of P-fimbriated pathogen species (27).

2.2.2. Other Anti-Pathogenic Mechanisms of HMO Mixtures

Group B *Streptococcus* (GBS), often associated with post-natal infection and mortality, and its interaction with different HMOs has been of significant scientific interest in recent years. Pooled HMOs were shown to inhibit GBS growth and biofilm formation, provoking an alteration in biofilm structure. A suggested reason for the antibiofilm activity of these oligosaccharides is interference with nutrient cross-membrane transport by adhesion to the pathogen exterior (21).

Additionally, pooled HMOs potentiated the bactericidal function of a select number of ribosome-targeting antibiotics, clindamycin and erythromycin especially, against antibiotic resistant GBS and Acinetobacter baumannii, without the protection of a biofilm (37). It was hypothesized that this is due to increased permeability of pathogens, a mechanism potentiated by polymyxins (38). Antibiotics inhibiting cell wall synthesis are not potentiated, while treating any pathogenic strain in combination with pooled HMOs.

Finally, pathogenic cellular invasion can be affected by the presence of an HMO mixture. Pooled HMOs can inhibit Escherichia coli invasion of epithelial bladder cells by over 80%. Reportedly, HMOs aid in the preservation of paxillin (39), which is associated with the promotion of cohesion of the epithelial cell monolayer as a focal adhesion molecule (40). The cell-protecting effect is further substantiated by complete inhibition of UPEC-induced upregulation of MAPK signalling (39), an important apoptotic cascade. Wider employment of this anti-pathogenic effect requires additional research with a higher variety of cell lines and experimental set ups.

Table 1. Overview of the anti-pathogenic functionalities of HMO Mixtures

HMO Characteristics (Source)	[HMO]	Strains Used	Observed Effects	References
Breast milk collected from first and fourth week of lactation	1:2 dilution	Gram-positive: *Streptococcus pneumoniae* Gram-negative: *Haemophilus influenzae*	Anti-adhesive effects against *Haemophilus influenzae* (HMWF) and *Streptococcus pneumoniae* (all HMOs)	(23)
Breast milk from healthy women collected 30 days after delivery	6 mg/mL	Gram-negative: UPEC	Anti-adhesive effects of neutral fractions (high > low Mw)	(28)
Colostrum (d1–4), transitional (d12–17) and mature (d28–32) breast milk from healthy women 25–	1200 µg/ well (50 µL)	Gram-negative: ETEC (CFA/I, CFA/ II fimbriae), UPEC (P, P-like fimbriae)	Inhibition of hemagglutination by desialylated fraction associated with binding to P-fimbriae	(27)
Pooled transitional breast milk samples	20 g/L	Gram-negative: *Neisseria meningitidis*	Inhibition of binding to pili by acidic HMO fraction	(35)
HMO fractions and modified HMO fractions from pooled human milk	1–2 g/L	Isolated, immobilized P-selectin	Interference acidic HMO fraction and P-selectin. Neutral HMOs show no interference.	(41)

Table 1. Continued

HMO Characteristics (Source)	[HMO]	Strains Used	Observed Effects	References
Colostrum (different fractions) collected four days after delivery	1, 5, and 10 mg/mL	Gram-negative: EPEC, *Vibrio cholerae*, *Salmonella fyris*	Anti-adhesive effects against Salmonella fyris (acidic, neutral, LMw), and Vibrio cholerae (neutral, hMw)	(17)
HMOs isolated from pooled human milk	15 mg/mL	Gram-negative: UPEC	Inhibition of bacterial invasion but no anti-adhesive effects, protection	(39)
Breast milk from healthy women collected between 3 days and 3 months postnatal	5 mg/mL	Gram-positive: GBS	Up to 40% growth inhibition	(21)
Breast milk from healthy women collected between 3 days and 3 months postnatal	5 mg/mL	Gram-positive: GBS (CNCTC, GB590, GB2)	8–32× MIC reduction with antibiotics in combination with HMOs	(42)
Breast milk from healthy women collected between 3 days and 3 months postnatal	5 mg/mL	Gram-positive: GBS, *Staphylococcus aureus* Gram-negative: *Acinetobacter baumannii*	GBS and *Staphylococcus aureus* biofilm inhibition, no antimicrobial effect	(43)
Breast milk from healthy women collected between 3 days and 3 months postnatal	5 mg/mL	Gram-negative: GBS (GB590, GB2)	HMO mixture more effective inhibition of pathogen growth and viability reduction than isolated oligosaccharides	(44)

2.3. Anti-Pathogenic Functionalities Isolated HMO Structures

The apparent antimicrobial functionalities of HMOs encouraged investigation of a number of isolated HMO structures. Even though the HMO mixture consists of over 100 distinct structures (45), the pathogenic interactions of only a relatively small number of individual HMO structures have been investigated (Table 2).

2.3.1. Neutral Isolated HMO Structures

An example of an isolated HMO widely studied for its interactions with pathogens is 2-fucosyl-lactose (2-FL). 2-FL (α–l–Fuc– (1→2) –β–d–Gal– (1→4) –d–Glc or, α–l–

fucopyranosyl– (1→2) –β–d–galacto–pyranosyl– (1→4) –d–glucopyranoside) is the most abundant fucosylated HMO in breast milk and has in multiple instances been linked with anti-adhesive properties. 2-FL mimics the H-2 epitope on epithelial cells. This glycosylic structure is important to pathogenic adhesion to epithelial cells (36). Through this mechanism, 2-FL was shown to inhibit the adhesion of *Campylobacter jejuni* (46), *Pseudomonas aeruginosa*, EPEC, *Salmonella enterica* (47), but not the adhesion of UPEC, *Vibrio cholerae* and *Salmonella fyris* (17). Even though specificity of certain pathogens for the H-2 epitope has been confirmed (48), the biochemical origin of pathogenic affinity for 2-FL was not further elucidated.

Although anti-adhesion functionalities of 2-FL have been well-described, there is scarce information about other types of anti-pathogenic effects of 2-FL. For interaction with pathogens in a biofilm structure, it is suggested that the neutral state of 2-FL limits it from entering the biofilm; attachment of a cationic element to 2-FL enables the molecule to enter extracellular polymeric substances (EPS) structures and exert antibiofilm activity (49)

3-Fucosyllactose (3-FL) is another trisaccharide observed in human milk, which is different in structure from 2-FL as its assimilation involves the enzymatic function of 3-fucosyltransferase (attachment of fucose to the reducing Glc end) instead of 2-fucosyltransferase (25). Like 2-FL, interaction with pathogens has been documented for 3-FL in a number of instances (17), though the concentration of 3-FL (0.44 g/L) is lower in human milk samples compared to 2-FL (2.74 g/L) [50]. 3-FL inhibits adhesion to a number of pathogens, including UPEC, Salmonella fyris (17), EPEC, *Campylobacter jejuni*, *Salmonella enterica* and *Pseudomonas aeruginosa* (47). Inhibition of adhesion of UPEC and *Salmonella fyris* by 3-FL, but not by 2-FL, indicates the importance of the location of fucosylation, apparently influencing the pathogenic receptor binding to the HMO structures (17). However, the alternative placement of fucosylation does not alter the antibiofilm activity, as 3-FL also seems unable to penetrate into biofilm structures (42).

Compared to the 2 most investigated HMO structures, 2-FL and 3-FL, isolated HMO structures of larger size tend to exert more anti-pathogenic characteristics. LNFP I for example, a monofucosylated LN(n)T isomer which carries its Fuc in an α1–2 linkage at the terminal Gal and is the second most prevalent HMO (after 2-FL) (50), shows a high anti-pathogenic potential. LNFP I can significantly reduce pathogenic growth of GBS, while also exerting some antibiofilm action against GBS. In comparison with the other LNFP (LNFP II and LNFP III), LNFP I exerts the strongest antimicrobial potential. In addition, the anti-pathogenic properties of single HMOs were found to be strain-specific (42).

2.3.2. Acidic Isolated HMO Structures

The isolated structures described thus far are neutral, which make up a large fraction of all oligosaccharides present in human milk (51). Sialylation of oligosaccharides produces a negatively charged entity under neutral conditions. However, this does not seem to

affect their ability to inhibit pathogenic adhesion; inhibition of UPEC and *Salmonella fyris* by 3-SL, a sialylated oligosaccharide structure, is comparable to inhibition by 3-FL [17]. 6-SL has been shown to be effective in inhibiting pneumocyte invasion *Pseudomonas aeruginosa* strains (52), while larger sialylated human milk oligosaccharides, such as LS-tetrasaccharide a (LSTa) exhibit a strong antimicrobial activity against GBS (44)

2.3.3. Fucosylated Oligosaccharides (FO)

Fucose is present in human milk and the proportion of fucosylated HMOs in term breast milk was recently reported as 35–50% (23). FO are constructed covalently joining of the l-fucose molecules to other monosaccharides via glycosidic linkages. l-Fucose is abundantly present in brown algae, like *Fucus*, *Laminaria*, *Sargassum*, and *Undaria* spp, as a major constituent of fucoidan (53). There is evidence supporting the inhibitory effects of fucoidan on *Helicobacter* infections by adhesion inhibition to mucosal surfaces (54). Another study included fractions of HMOs, containing about 5–20 different high-mass glycans with different degrees of fucosylation, in a neoglycolipid array (55) and demonstrated that high-mass HMOs with oligovalent fucose can exhibit stronger binding capacities towards blood group—active mucin-type O-glycans compared with monovalent fucose HMOs. Furthermore, HMO fractions with the strongest binding capacities contained hepta-to decasaccharides expressing branches with terminal Lewis-b antigen or blood group H1 (55,56). It has been recently proved that the presence of fucose alone does not correlate to antimicrobial activity, while the location and degree of fucosylation does play a key role in HMO antimicrobial activity (42).

Table 2. Overview of the anti-pathogenic functionalities of isolated HMOs.

HMO Characteristics	[HMO]	Strains Used	Observed Effects	References
2-FL, 3-FL, 3-SL,	6-SL 2-FL = 2.5 mg/mL 3-FL = 0.5 mg/mL 3 0 -SL = 0.1 mg/mL 6 0 -SL = 0.3 mg/mL	EPEC, *Vibrio cholerae*, *Salmonella fyris*	Anti-adhesive effect of 6-SL and 3-FL against *Escherichia coli* and *Salmonella fyris*	(17)
Synthesized 2-FL, 3-FL	10 mg/mL	Gram-negative: *Campylobacter jejuni*, EPEC, *Salmonella enterica*, *Pseudomonas aeruginosa*	Differential anti-adhesive effect	(47)
3-SL and 6-SL	2 µg/mL–1 mg/mL	Gram-negative: *Pseudomonas aeruginosa*	Dose-dependent inhibition by 6-SL of pneumocyte invasion (lung)	[52]

Table 2. Continued

HMO Characteristics	[HMO]	Strains Used	Observed Effects	References
3-SL, 6-SL, LNT, LSTa, LSTc, DSLNT	5 mg/mL	Gram-positive: GBS	Inhibition of biofilm production and growth by larger sialylated oligosaccharides	(52)
2-FL, 3-FL, DFL, LNT, LNnT, LNFP I, LNFP II, LNFP III, LNT II, para-LNnH, LNnH	5 mg/mL	Gram-positive: GBS	Strain-specific antimicrobial activity, no biofilm inhibition, fucose not involved in antimicrobial function	[42]
2-FL1-N-2-FL	-	Gram-positive: GBS	No antimicrobial or antibiofilm activity of 2-FL Antimicrobial/antibiofilm activity due to cationic moiety of 1-N-2-FL	(42)

3. Alginate Oligosaccharides

3.1. Structure

Alginate is a biopolymer, present in the cell walls of brown algae, and is composed of a sequence of two types of monosaccharides, 1,4–linked β–d–mannuronic acid (M) and 1,4 α–l–guluronic acid (G) (Figure 2) (57). The M/G monosaccharide ratio, expressed as guluronic content (GC), is an important indication for antimicrobial functioning, as the G monomer has been shown to be preferred for cationic interaction as it is negatively charged (58). From these polymers, AOS can be derived through enzymatic depolymerization or acid hydrolysis. So far, alginate biosynthesis has been detected in the *Azotobacter vinelandii* and the *Pseudomonas* species (59). In *Pseudomonas aeruginosa* specifically, alginate biopolymers are an essential component of the biofilm EPS (60). Alginates in these biofilms have slightly divergent structural characteristics, as they do not contain multiple G monosaccharides in sequence, termed G-blocks (61).

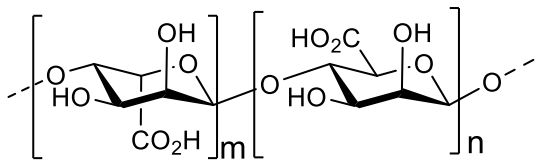

Figure 2. Structure of the main components of AOS; 1,4-linked β-D-mannuronic acid and 1,4-linked α-L-guluronic acid.

3.2. Anti-Pathogenic Functionalities

AOS have many antimicrobial functionalities (Table 3), and three main mechanisms of antimicrobial potential can be identified, all of which affect biofilm growth and development. First, AOS inhibit pathogenic swarming motility and proliferation. Second, AOS elicit a Ca^{2+} chelating effect in the presence of bio-alginates. Finally, AOS affect expression of *quorum-sensing (QS)* genes. Importantly, most of the anti-pathogenic effects of AOS have been elucidated studying its effect on AOS have been elucidated studying its effect on *Pseudomonas aeruginosa*, due to the alginate presence in the *Pseudomonas aeruginosa* biofilm composition.

3.2.1. Biofilm Inhibition

AOS have an extensive biofilm-inhibiting function. They inhibit pathogenic swarming and motility, important mediators in biofilm formation (62), in Gram-negative pathogenic strains *Pseudomonas aeruginosa*, *Escherichia coli* and *Proteus mirabilis* (63,64). In *Pseudomonas aeruginosa*, inhibition of motility appears to be caused by AOS adhesion to the pathogenic exterior and flagella, along with a zeta-potential of the pathogenic cell surface by anionic AOS (65,66). Inhibited motility and the resulting cellular aggregation has an inhibiting effect on the formation and growth of biofilms (67). Even though alteration of surface-charge by AOS is limited to Gram-negative bacteria due to the polyanionic nature of the LPS layer of Gram-positive strains, AOS interaction with the LPS layer does induce biofilm-destructive bacterial aggregation of *Streptococcus mutans* (67). Additionally, swarming of pathogenic cells and structured biofilm formation play an important role in the development of antibiotic resistance (68). Indeed, AOS increase efficacy of several antibiotics against multidrug-resistant *Pseudomonas aeruginosa* (63,66,69). Synergistic functionalities of AOS are not limited to antibiotics, as AOS adhesion to bacterial surface was also found to decrease colonization and biofilm formation in combination with an antibacterial and antifungal agent, triclosan (70).

3.2.2. Metal ion Scavenging

AOS are potent Ca^{2+} scavengers. The Ca^{2+} scavenging activities of AOS inhibit biofilm formation in a number of ways. Ca^{2+} crosslinks alginate biopolymers, one of the major components of the EPS, improving structure and stability of the biofilm (71,72) and contributing to *Pseudomonas aeruginosa* resistance to antibiotics and elements of the immune system (73,74). By scavenging alginate-associated Ca^{2+} in the biofilm, AOS remove these crosslinks and compromises EPS integrity and increases susceptibility of the biofilm to antibiotic treatment (58,63,66,69), which is in accordance with the observed higher affinity of Ca^{2+} for G-rich AOS (58). Furthermore, considering Ca^{2+} availability induces alginate production *Pseudomonas aeruginosa* (75), AOS could also have a mediating function in the process of alginate-synthesis. The chelating properties are not universal to all bivalent cationic metals. Fe^{2+} for example, is another important factor in formation of the *Pseudomonas aeruginosa* biofilm and alginate production (76,77). Contrary to Ca^{2+}, Fe^{2+} is scavenged by neither AOS nor alginate (78).

3.2.3. *Quorum Sensing (QS)* System Inhibition

Finally, AOS inhibit expression of *QS* genes in *Pseudomonas aeruginosa*. *QS* signaling is a cell-to-cell communication through extracellular exchange of signaling molecules to coordinate pathogenic behavior (79). The system is responsible for bacterial adaptation to the environment and plays a role in biofilm formation, swarming behavior and antibiotic resistance of *Pseudomonas aeruginosa* (80). Additionally, through *QS* signaling, biofilm structure and integrity is influenced, for example through production of eDNA (81), an important component of the EPS of *Pseudomonas aeruginosa*, acting as a cellular connector (82,83). AOS inhibit production of two of the main components of the *QS* signaling system, acyl homoserine lactones (AHL) and C4–AHL and 3–oxo–C12–AHL (84). This effectively inhibits pathogenic swarming motility and biofilm– formation. As AOS do not show specific interactions with DNA (69) modulation of *QS* signaling molecule expression is believed to be achieved through interaction between AOS and C4–AHL and 3–oxo–C12–AHL. Consequently, the decline in intercellular signaling results in decreased synthesis of several virulence factors, such as elastase and pyocyanin (84). Virulence factors exhibit important functionalities in biofilm persistence and antibiotic resistance (85). Pyocyanin specifically inhibits production of eDNA (86). Noticeably, AOS are able to make bacterial strains more susceptible to H_2O_2 by inhibiting *QS*-controlled virulence factors. *QS* affects the *Pseudomonas aeruginosa* resistance to H_2O_2 by production of antioxidants, such as superoxide dismutase and catalase, leading to the *Pseudomonas aeruginosa* resistance to the toxic free oxygen radicals (87,88).

Table 3. Overview of the anti-pathogenic functionalities of AOS.

AOS Characteristics	[AOS]	Strains Used	Observed Effects	References
GC: 90–95% (OligoG CF 5/20), 46%, 0% MW: 2.6 kDa	2%, 6%, 10%	Gram-negative: *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Escherichia coli*	Inhibition of motility and biofilm formation, antibiotic synergy	(63)
OligoG CF-5/20	2%, 6%, 10%	Gram-negative: *Pseudomonas aeruginosa,*	*Acinetobacter baumannii* Structural interference biofilm formation, antibiotic synergy	(64)
OligoG CF-5/20	2%, 6%, 10%	Gram-negative: *Porphyromonas gingivalis*	Gram-positive: *Streptococcus mutans Triclosan* synergy	(70)
OligoG CF-5/20	0.2%–10%	Gram-negative: *Pseudomonas aeruginosa*,	*Burkholderia* spp. Inhibition of pathogenic cell motility	(65)
Alginate-derived oligosaccharides M/G: 2.28 MW: 300 kDa	2 mg/mL	Gram-negative: *Pseudomonas aeruginosa*	Antibiotic synergy, anti-biofilm effect, decrease in virulence, increase in susceptibility to H_2O_2 of pathogen	(88)

Table 3. Continued

AOS Characteristics	[AOS]	Strains Used	Observed Effects	References
OligoG CF-5/20	5–100 mg/mL	Gram-negative: *Pseudomonas aeruginosa* Gram-positive: *Streptococcus mutans* Cellular aggregation of *S. mutans* and *P. aeruginosa* and binding of OligoG CF-5/20 to *P. aeruginosa.*	Anti-microbial effects are not related to structural alterations in LPS or cell permeability	(67)
OligoG CF-5/20	2%	Gram-negative: *Pseudomonas aeruginosa*	Antibiotic synergy	(66)
OligoG CF-5/20 -		Gram-negative: *Pseudomonas aeruginosa*	Inhibition of QS-signaling	[84]
OligoG CF-5/20	0.5%, 2%, 6%	Gram-negative: *Pseudomonas aeruginosa*	Disruptive effect on biofilm formation, established biofilm	(69)

4. Chito-Oligosaccharides/Chitosan Oligosaccharides

4.1. Structure

One of the most extensively investigated oligosaccharides are COS. COS are enzymatically or chemically processed products of chitin or chitosan polymers. Chitin is abundantly present in crustacean or arthropodic shells, while chitosan is more rare and must be extracted from cell walls of specific fungi (89). Crustacean and arthropodic chitin consists of β–1,4–linked N-acetyl-d-glucosamine (GlcNAc), whereas chitosan consists of GlcNAc and the deacetylated form β–1,4–linked d–glucosamine (GlcN) (Figure 3). Chitin and chitosan copolymers are distinguished based on their DA: a DA of >70% usually refers to chitin, whereas a DA of <30% refers to chitosan (90). The large chitin or chitosan polymers extracted from these biological sources, can be chemically or enzymatically hydrolysed into COS, with DP <20 and molecular weight of <3900 Da (91,92). COS is highly soluble in a slightly acidic pH due to the charged state of the amine moiety (cationic nature) (93–95). Chemically or enzymatically transformed COS is highly heterogenous with respect to DP and DA while the acetylation pattern (AP) can only be controlled to a certain extent (92,96). Their enzymatic transformation allows for a limited control of the acetylation pattern (97). GlcNAc is a ligand of F1C fimbriae in UPEC strains, involved in adhesion (98). Nevertheless, antimicrobial effects of chitosan-based COS have been more extensively studied than chitin-based COS related to their increased solubility and cationic nature, making them more viable pharmacological prospects (90,99,100).

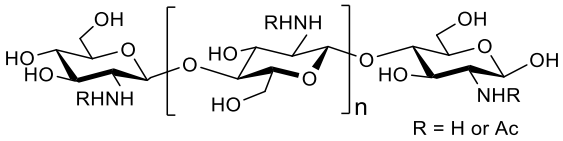

Figure 3. Structure of the main components of COS; with the monosaccharides N-acetylglucosamine (GlcN) and GlcNAc.

4.2. Anti-Pathogenic Functionalities

Ever since the antimicrobial activities of chitosan were first recognized (101), a wide range of studies have been conducted aiming to elucidate different antimicrobial pathways. A summary of the key antimicrobial activities of COS is presented in Table 4. First, COS have the potential to disrupt the bacterial cell membrane. Additionally, the surface-associating properties of COS can inhibit adhesion of pathogenic bacteria to host cells. COS also exhibit some antibiotic-potentiating properties and can inhibit RNA transcription in Gram-negative species. Importantly, different anti-pathogenic effects elicited by COS are often ambiguous amongst different sources. For example, the antimicrobial characteristics of COS are stronger against Gram-negative (95,102–104) or Gram-positive (93,94,105) bacterial strains, depending on the source.

4.2.1. Cell Membrane Disruption

Polymeric molecules bearing a cationic charge are known to adhere to Gram-negative bacterial cell surfaces by ionic interactions with anionic lipopolysaccharide patterns (106). Polyethylenimine, for example, is of great interest in pharmaceutical research for its polycationic functionalities and capability (107,108). For cationic glucosamine components, present in COS, a similar mechanism is proposed, creating an impermeable cationic oligosaccharide layer around the bacteria (102,109) Concomitantly, chitosan adherence to bacterial cell surface promotes leakage of electrolytes and metal ions from the bacterial lumen (110–112) Metal ions and other nutrients essential for bacterial proliferation are unable to diffuse across the bacterial membranes (102,112). Prolonged exposure to COS and the resulting osmotic imbalance results in inhibition of growth, cell swelling and, ultimately cell lysis (112,113). The bactericidal activity of COS increases with an increased glucosamine share and is greater than that of polymeric chitosan (114).

4.2.2. Adhesion Inhibition

unlike other types of oligosaccharides, the anti-adhesive properties of COS have been relatively poorly studied. Yet, COS (DP 4 >12, DA 15–65%) was found to be a potent, pathogen-specific inhibitor of EPEC adhesion, but not verotoxin-producing Escherichia coli (VTEC) (115,116). Interestingly although EPEC expressing F1C fimbriae were demonstrated to show affinity for GlcNAc, the DA hardly influences inhibition of pathogenic adherence (115). An explanation for this is the abundance of strong ionic interactions mediated by the cationic amine moiety of glucosamine in addition to the GlcNAc recognition.

4.2.3. Association with Bacterial DNA

Chitosan has a strong interaction with fungal and plant DNA, mediated by electrostatic interactions, resulting in inhibited mRNA transcription (117–120). Chitosan only penetrates into bacterial cells after disruption/lysing of the bacterial membrane (112,113). COS, on the other hand, is linked to binding to bacterial DNA independent of bacterial lysis, inhibiting DNA transcription. This effect is most potent with COS Mw ≤ 5000 (121). At this size, COS is small enough to penetrate the bacterial membrane (94,122). COS interference with DNA transcription is linked to a decreased alginate production and, thus, biofilm formation in Pseudomonas aeruginosa (88). mRNA transcription in Gram-negative species, however, is not affected in a similar manner, as their thick peptidoglycan layer prevents cellular penetration of COS (94,104).

4.2.4. Synergy with Antibiotic Treatment

Multi-drug resistance (MDR) is often achieved by bacteria by upregulation transmembrane multidrug efflux pumps (123). COS sensitize multi-drug resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* to a number of common antibiotic formulations (88,124,125), possibly through formation of the aforementioned cationic oligosaccharide layer around bacteria, or ionic interactions with multidrug efflux pumps.

Table 4. Overview of the anti-pathogenic functionalities of COS.

COS Characteristics + Source	[COS]	Strains Used	Observed Effects	References
Chitosan oligosaccharides-from chitosan with DA 89%, DP 3–6 (805)	0.01–0.5%	Gram-negative: *Escherichia coli*	Antibacterial activity (anti-growth) and 0.5% completely inhibited the growth of *E. coli*	(126)
Chito-oligosaccharides DA 8.5%, Mw 2–30 kDa	0.10%	Gram-negative: *Aggregatibacter actinomycetemcomitans* Gram-positive: *Streptococcus mutans*	Pathogenic membrane disruption	(102)
Chito-oligosaccharides DA 11% Mww <10, <5, <1 kDa	0.1–1%	Gram-negative: *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa* Gram-positive: *Streptococcus mutans, Micrococcus luteus, Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis*	Higher anti-microbial (anti-growth) effect high-Mw COS	(105)

Table 4. Continued

COS Characteristics + Source	[COS]	Strains Used	Observed Effects	References
Chitosan Mw 5, 8 kDa	0.01–0.5%	Gram-negative: *Escherichia coli*	mRNA transcription inhibition	(121)
Chitosans (Mw = 1671, 1106, 746, 470, 224, and 28 kDa) Chitosan oligomers (Mw = 22, 10, 7, 4, 2, and 1 kDa)	1%	Gram-negative: *Escherichia coli, Pseudomonas fluorescens, Salmonella typhimurium, Vibrio parahaemolyticus* Gram-positive: *Listeria monocytogenes, Bacillus megaterium, Bacillus cereus, Staphylococcus aureus*	Higher anti-microbial effect (anti-growth) of chitosan compared to COS Chitosan showed stronger bactericidal effects for gram-positive bacteria than gram-negative bacteria	(93)
Chitosan Mw < 5 kDa	0.25–1%	Gram-negative: *Escherichia coli* Gram-positive: *Staphylococcus aureus*	*E. coli* growth inhibition (lower Mw more effective) S. aureus growth inhibition (higher Mw more effective)	(94)
Chito-oligosaccharides DA 35.2–37.8% DP 1–6	0.1–0.5%	Gram-negative: *Escherichia coli* Gram-positive: *Bacillus cereus*	Growth inhibition and cell membrane disruption	(112)
Chito-oligosaccharides DA 3%, DP ∼4	0.25–2.5%	Gram-negative: VTEC, EPEC, *Desulfovibrio desulfuricans*	Selective anti-adhesion properties	(116)
Chitosan oligosaccharides DA 98.8%, DP 1–16	0.0001–0.5%	Gram-positive: *Staphylococcus aureus*	Cell membrane lysis	(117)
Chito-oligosaccharides DA 15–20%, MW<5, <3kDa	1–5% Gram-negative: *Escherichia coli*	Gram-positive: *Staphylococcus aureus*	Antimicrobial effect (anti-growth) on Escherichia coli, but not Staphylococcus aureus	(104)
Chitosan DA > 90%	0.0004–6.7%	Gram-negative: *Pseudomonas aeruginosa* (MDR)	Synergy with sulfamethoxazole treatment (anti-growth effect)	(125)
Chitosan oligosaccharides-Mw= 10,000 Da and 1000 Da)-from chitosan with DA 90–95%	0.5–10 mg/mL	Gram-negative: *Vibrio vulnificus*	Higher antimicrobial effect (anti-growth) of water-soluble COS with high molecular weight	(127)

Table 4. Continued

COS Characteristics + Source	[COS]	Strains Used	Observed Effects	References
Chitosan DA >90%	0.0004–6.7%	Gram-possitive: *Staphylococcus aureus* (MDR)	Synergy with several AB treatments (anti-growth effect)	(124)
Chitosans DA 80–85% Mw = 628, 591 and 107 kDa Chito-oligosaccharides DA 80–85% Mw =<5and <3 kDa	0.5%	Gram-negative: *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* Gram-positive: *Staphylococcus aureus, Staphylococcus epidermidis*	Higher antimicrobial effect (anti-growth) of the 3 chitosans	(104)
Chitin (DA 35, Mw 388 Da) Chitosan (DA 80, Mw 12 Da) COS	0.003–0.1% *Staphylococcus aureus*, *Bacillus subtilis, Bacillus cereus*	Gram-negative: *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Vibrio cholerae, Shigella dysenteriae, Enterobacter agglomerans, Prevotella melaninogenica, Bacteroides fragilis* Gram-positive: *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus*	Higher antimicrobial effect (anti-growth) COS compared to biopolymers	(114)
Chito-oligosaccharides DA ~65%, DP 3–5	2%	Gram-negative: *Pseudomonas aeruginosa*	Anti-growth, anti-biofilm functionalities and synergy with azithromycin	(88)
Chito-oligosaccharides DA 9–14%, DP<5-30	1–10%	Gram-negative: *Escherichia coli* Gram-positive: *Listeria monocytogenes*	High antimicrobial effect (anti-growth) with high DP	(103)
Chito-oligosaccharides- from chitosan with DD 80 and 90%- from chitosan with Mw = 5.1, 14.3 and 41.1 kDa	0.002–0.064%	Gram-negative: *Escherichia coli, Salmonella typhimurium, Salmonella enteritidis*	High antimicrobial effect (anti-growth) with low DP, potent ferrous chelating activity at low DP	(128)

5. Fructo-Oligosaccharides

5.1. Structure

FOS are a common component of a healthy diet (129) and are widely investigated for their prebiotic functionalities. Sucrose (a glucose-fructose disaccharide) is transformed into fructose by a transfructosylating enzyme. The FOS structure is characterized by a single sucrose monomer followed by a variable number of fructose monomers, sometimes in a 2→6 but often with a 2→1 linkage (Figure 4). FOS structures larger than DP10 are termed inulin.

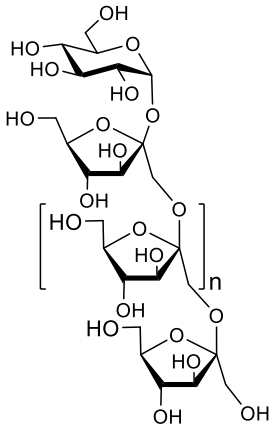

Figure 4. Structure of the main components of FOS; a glucose monomer, followed by an n number of fructose monomers in sequence.

5.2. Anti-Pathogenic Functionalities

Although FOS are renowned for their indirect anti-pathogenic functionalities, namely their prebiotic capacity, direct anti-pathogenic functionalities of FOS are not widely investigated. FOS and inulin were associated with pathogenic anti-adhesion. Inulin inhibited the adhesion of Escherichia coli to human epithelial cells and FOS decreased the ability of *Escherichia coli* Nissle 1917 to adhere to human intestinal epithelial cells (130,131). In addition, FOS decreased the growth, biofilm formation, and motility behaviour of *Pseudomonas aeruginosa* PAO1, while inulin showed the opposite effects. The FOS-induced decrease in exotoxin A, a *P. aeruginosa* virulence factor, could be a possible mechanism for the reduction in pathogenicity (132).

A number of causes may potentially underlie the scarcity of the anti-pathogenic functionalities of FOS. As we have seen, an oligosaccharide can serve as a substrate for a bacterial adhesin involved in pathogenic adhesion and offers a predictive value for the anti-adhesive functionalities. So far, FOS has not been associated with targeting any specific pathogenic adhesins and the existence has only been theorized (131). Secondly, unlike several other oligosaccharides, FOS lack functional groups capable of bearing a charge. For this reason, FOS do not engage in ionic interaction the way (partially) charged oligosaccharides.

6. Galacto-Oligosaccharides

6.1. Structure

Although galactose is an important monosaccharide component of some HMOs, GOS are not a component of HMOs (25), but are known to mimic the biological effects of HMOs (133). Commercially available GOS are most commonly composed of β–galactooligosaccharides instead of α–galactooligosaccharides and usually have a DP ranging from 2 to 6 (134). Typically, commercial GOS mixtures are structurally heterogenous due to enzyme activity, as they feature different types of linkages between monosaccharides. Most of these linkages are of 1→4 (Figure 5) or 1→6 in nature. Often, the enzymes used produce different types of linkages within one oligosaccharide, resulting in a range of different oligosaccharides in a mixture (135). Unfortunately, many experiments performed with GOS have no clear characterization of the linkages of the used oligosaccharide mixture, and often an indication of the suspected GOS linkages are provided as a suggestion. Additionally, other types of oligosaccharides can be galactosylated, adding galactose characteristics.

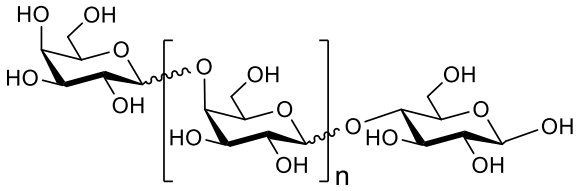

Figure 5. Structure of the main components of GOS; 1,4–linked and 1,6–linked β–galactose and a reducing-end glucose.

6.2. Anti-Pathogenic Functionalities

Two mechanisms of antimicrobial activity by GOS have been identified. GOS elicit anti-adhesive properties but can also inhibit host cell interaction with pathogenic toxins (Table 5).

6.2.1. Adhesion Inhibition

GOS interactions are attributed to their association to specific pathogenic adhesins. Some parasites, such as Entamoeba histolytica, use β–galactose patterns on intestinal epithelial cells for lectin mediated adhesion (136,137). For this reason, GOS have been subject to investigation for the identification of a similar effect (138,139). GOS were first reported to reduce cellular adhesion of EPEC (138), and later the anti-adhesive effect of GOS was shown for a number of other pathogenic strains, such as *Salmonella typhimurium* (139). The anti-adhesive effect of GOS on *Citrobacter rodentium* is dependent on adhesin expression, as deduced from a diminished antiadhesive effect of GOS after expression alteration of fimbria-mediated genes (140). This further suggests an adhesin-specific anti-adhesive effect of GOS. However, GOS did not show an anti-adhesive and anti-growth effect against *Listeria monocytogenes* (141). Until now, a specific interaction between GOS and a pathogenic adhesin has not been identified. Interestingly, GOS significantly inhibit cellular

adhesion of *Cronobacter sakazakii* (142), a strain suggested to exert a fimbria-independent mechanism of cellular adhesion (143). Therefore, the anti-adhesive activity of GOS could (at least in part) be fimbria-independent.

6.2.2. Anti-Toxin Binding

Cholera toxin (Ctx) is produced and excreted by the Vibrio cholerae strain and binds to host cell surface GM-1 receptors, causing cellular salt and H2O excretion, resulting in diarrhea (144,145) GM-1 receptors are lipid-conjugated oligosaccharides and contain a terminal galactose (146). The GM-1 receptor, expressed on the membrane of intestinal epithelial cells, is responsible for Ctx entry into the host cell (147), although fucose binding is now also considered to be part of this (148). GOS is hypothesized to bind to Ctx, inhibiting its GM-1 host cell entry mechanism (149) similar to dendritic GM1-oligoscaccharide compounds (150).

Table 5. Overview of the anti-pathogenic functionalities of GOS

GOS Characteristics	[GOS]	Strains Used	Observed Effects	References
DP 3–7	0–32 mg/mL	Gram-negative: EPEC	Anti-adhesive effect	(138)
DP 3–6	1.56–100 mg/mL	Gram-negative:	Vibrio cholerae Anti-Ctx	(149)
DP 1–4	2.5 mg/mL	Gram-negative: *Salmonella typhimurium*	Anti-adhesive and anti-invasive effect	(139)
DP 2–6	20 mg/mL	Gram-positive: *Listeria monocytogenes*	No anti-adhesive and anti-growth effec	(142)
-	10–50 mg/mL	Gram-negative: *Citrobacter rodentium*	Anti-adhesive effect	(140)

7. Mannan-Oligosaccharides

7.1. Structure

There are multiple ways of producing mannan-oligosaccharides (MOS). Previously, yeast products were harvested and directly applied for *in vitro* experimentation (151,152). Nowadays, the most common ways of MOS production are chemical synthesis or autolysis of biopolymers extracted from yeast. Synthetically produced MOS can be structurally defined and nature of intergycosidic linkages can be determined (153). Alternatively, isolation of autolysed yeast cell wall yields a heterogenous mixture of (branched) MOS, including 1→2, 1→4 and 1→6 d–mannose linkages (154). The structure of MOS is shown in Figure 6. A drawback of MOS for pharmacological purposes is its branched nature and the unpredictability of the product structure after enzymatic production.

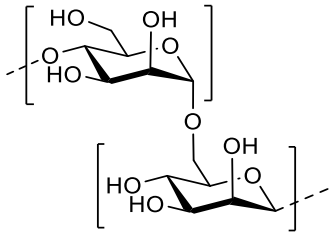

Figure 6. Structure of the main components of MOS; 1→4 linked D–mannose (top) and 1→6 linked D–mannose (bottom).

7.2. Anti-Pathogenic Functionalities

The mannose monosaccharides and MOS are well-known for their anti-adhesion capacity against pathogen adhesion, as summarized in Table 6.

Adhesion Inhibition

The mannose monosaccharide is an established and widely studied ligand for the FimH domain of type I fimbriae. The FimH domain of the type I fimbria is responsible for recognition of mannose patterns on host cell exterior and subsequent mannose-dependent pathogenic adhesion (155,156). Type I fimbriae are commonly found in *Salmonella* spp. and *Escherichia coli* and play an important role in adhesion by binding to mannose patterns in host cell epithelial receptors (151,152,157) It was previously shown that glycosides of mannose exhibit amplified anti-adhesive properties towards Escherichia coli compared to mannose monosaccharides, indicating the importance of a hydrophobic region in the vicinity of the mannose binding area for type I fimbria adhesion (158,159). Mannose can bind to different FimH variants from different *Escherichia coli* pathotypes, concluding that mannose affinity for the FimH domain is independent of pathotype (160). The mannose binding pocket of the FimH was later determined to be identical within different pathogenic species, including *Escherichia coli* and *Klebsiella pneumonia* (161). As mannose glycosides have a significantly higher affinity for the *Klebsiella pneumonia*, it is likely that FimH structure varies between species and are also presented differently (161). This difference is also reflected in superior Escherichia coli specificity for mono-or trimannose moieties (162,163). Furthermore, significant reduction in the adherence of *Campylobacter jejuni* and coli to human epithelial cells was observed in the presence of MOS (163). An overview of anti-adhesion activities is shown in Table 6. MOS binds to the FimH domain in competition with mannose patterns on host epithelial cells. This inhibits pathogenic adhesion by exerting a receptor-mimicking function (164,165). Contrary to the mannose monosaccharide described earlier, addition of hydrophobic triethylene glycol to MOS (DP ≥ 3) does not increase the affinity for FimH compared to unconjugated MOS (160). Inhibition of pathogenic adhesion by MOS is non-superior to inhibition by yeast cell wall, containing mannos biopolymers (166).

Table 6. Overview of the anti-pathogenic functionalities of MOS.

MOS Characteristics	[MOS]	Strains Used	Observed Effects	References
DP 2–6	0.1–0.5 mM	Gram-negative: *Escherichia coli*	Anti-adhesive effect	(167)
DP 9	25 µM	Gram-negative: *Enterobacter cloacae*	Anti-adhesive effect	(168)
DP 3 MOS	0.13 M–087 M	Gram-negative: *Escherichia coli*	Affinity for FimH mannose > MOS	(160)
Partially purified yeast MOS and soluble supernatant fraction of MOS	10–50 mg/mL	Gram-negative: *Campylobacter jejuni, Campylobacter coli*	Anti-adhesive effects	(163)
Yeast MOS	6 mg/mL	Gram-negative: *Escherichia coli, Salmonella pullorum*	Anti-adhesive effect (less effective than yeast cell walls)	(166)

8. Pectic Oligosaccharides

8.1. Structure

Pectin is a plant biopolymer, acting as a stabilizer for the cellulose network (169). Pectin is a complex biopolymer made up of combined monosaccharides, most importantly (1→4) linked d–galacturonic acid (GalA). GalA is the main component of the pectin backbone, with a L-rhamnose content of 2–4% (Figure 7) (170). GalA monosaccharides of pectin biopolymers are 6–methyl esterified to a certain extent, depending on the presence of pectin esterase in the source (171). Much like acetylation of chitosan, the extent to which pectin is methylated dictates its function; low methylation ensures higher hydrophilicity and more interaction with cationic metal agents (172). Depolymerization of pectin yields POS, which can due to the high diversity of the source polysaccharide, assume a high variety of forms. POS investigated for antimicrobial purposes are often depolymerized by enzymatic hydrolysis and are often derived from orange/bergamot peel (173,174). Pectin found in bergamot peel is especially useful due to presence of 'hairy' and 'smooth' regions of GalA backbone. Hairy regions of pectin are equipped with arabinose, galactose, glucose, mannose and xylose elements (175). This way, a large number of structurally different oligosaccharides can be synthesized from a single source.

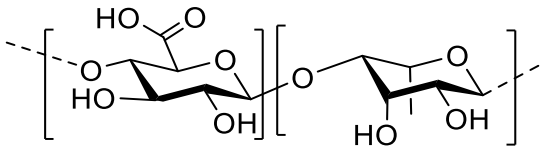

Figure 7. Structure of the main components of POS; a non-methylated D–galacturonic acid monomer (left) linked in a β1→4 fashion with a rhamnose monomer (right).

8.2. Anti-Pathogenic Functionalities

POS have a wide range of antimicrobial activity as summarized in Table 7. POS have the capability to inhibit growth of several pathogens, inhibit adhesion of pathogenic bacteria and can also interact with pathogen-produced Shiga-like toxins (Stx).

8.2.1. Growth Inhibition

Although multiple types of POS have shown to inhibit pathogenic growth, no mechanism has been determined thus far. Citric POS inhibit growth of a number of pathogenic strains, with superior efficacy of growth inhibition of low Mw POS for all inhibited strains (176). The mechanism through which inhibition is achieved seems to be strain-dependent, as Gram-negative *Campylobacter jejuni* growth remains unaffected (177). POS extracted from haw fruit show inhibition of pathogen growth at relatively low concentrations compared to citrus-extracted POS, although the mechanism through which inhibition is achieved remains unclear (178). The induced strain-dependent growth inhibition could be related to increased radical scavenging abilities of charged POS. However, unlike COS and AOS, unspecific ionic interaction of charged carboxylic acid groups with the pathogenic exterior is unlikely to be the main source of pathogenic growth inhibition by POS, as it is strain-dependent.

8.2.2. CO2 Radical Production

POS have shown to efficiently scavenge free radicals (179,180). Free radicals, such as HO•, have a number of pathological effects, such as DNA damage and carcinogenesis (181,182). Radical-scavenging substances have been widely studied for their attractive pharmacological properties (183). Interestingly, HO• radical scavenging appears to trigger $CO2•^-$ radical production by several different types of POS, which is hypothesized to inhibit *Staphylococcus aureus* and, less significantly, *Escherichia coli* growth (184). However, due to the versatility of POS characteristics, the anti-pathogenic effect of $CO2•^-$, if any, has not been successfully gauged.

8.2.3. Adhesion Inhibition

POS inhibit adhesion of several kinds of pathogenic strains, most notably, *Escherichia coli*. The anti-adhesive mechanism referred to is inhibition of P-fimbria-mediated adhesion (185–187). The extent of inhibition exhibited by POS seems to be beneficially influenced by high GalA content and low degree of 6-methyl esterification (188). However, the P-fimbriae-specific inhibition of adhesion is not supported by the presence of a GalA binding pocket on these P-fimbriae (186,188). For this reason, the exact mechanism of fimbria-specific inhibition of adhesion is still unclear. Additionally, high uronic acid content in POS and, consequently, higher ionic interactions between oligosaccharides and pathogens have been proposed to contribute to the anti-adhesion functionalities against Gram-positive *Staphylococcus aureus* bacteria. Interestingly, uronic acid-rich oligosaccharides did not prove effective for inhibition of adhesion of *Escherichia coli* (189).

Table 7. Overview of the anti-pathogenic functionalities of POS.

POS Characteristics + Source	[POS]	Strains Used	Observed Effects	References
Mw 1–4 kDa Citrus (high methylation) Apple (low methylation)	10 mg/mL	Gram-negative: *Escherichia coli* O157:H7 Shiga toxin	Inhibition of host cell infiltration of Stx	(185)
Mw 1–12 kDa Panax ginseng	0.01–0.5 mg/mL	Gram-negative: *Aggregatibacter actinomycetemcomitans* Gram-positive: *Staphylococcus aureus*	Anti-adhesive effect	(189)
DP 2–3 Orange peel	2.5 mg/mL	Gram-negative: EPEC, VTEC,	Desulfovibrio desulfuricans Anti-adhesive effect	(186)
GalA:Rhamnose 1:1 Albedo of orange peel	0.05–2.5 mg/mL	Gram-negative: *Campylobacter jejuni* Inhibited	Caco-2 cell invasion	(177)
0.2–6 kDa 93.6% Uronic acid Haw	1–10 mg/mL	Gram-negative: *Escherichia coli*	Antimicrobial activity dependent on concentration and low pH	(178)
DP 6–19 Orange peel	1–100 mg/mL	Gram-negative: *Escherichia coli* Gram-positive: *Staphylococcus aureus, Bacillus subtilis*	Antimicrobial activity-low Mw more effective	(176)
Apple, citrus, polygalacturonic acid	0.1 mg/mL	Gram-negative: *Escherichia coli* Gram-positive: *Staphylococcus aureus*	Growth inhibition, potentially through CO2 radical production	(184)
Mw 9–73 kDa Orange peel	0.005–5 mg/mL	Gram-negative: Shigatoxigenic *Escherichia coli*	Anti-adhesive effect, direct interaction with Stx	(188)

8.2.4. Inhibition of Toxin-Binding

Shigatoxigenic *Escherichia coli* produce Stx type 1 as well as type 2. The pentamer subunit of Stx, termed StxB interacts with a number host cell surface constituents, the main one globotriaosylceramide (Gb3), for epithelial cell internalization (190,191). The Gb3 receptor is a lipid-conjugated oligosaccharide structure consisting of a Galα1,4Galβ1,4Glc trisaccharide (192,193). Even though Stx type 1 and type 2 do not necessarily always use the same pathways to enter cells (193), POS inhibit the host cell uptake of both types of

Shiga toxin in two ways. First, GalA-rich POS is associated with competitive binding of Gb3 with Shiga toxin. GalA inhibitory capacity of Gb3 is similar to that of its primary substrate (188). Additionally, POS directly binds to Stx, due to structural similarities between POS and the galabiose receptor. This interaction inhibits Stx association to the Gb3 receptor, to a comparable extent to inhibition by the minimum receptor analogue galabiose (Galα1,4 Gal) (194), reducing host cell uptake of Stx (174). Similarly, the Stx-binding capabilities of POS have been proposed to assist in inhibiting *Campylobacter jejuni* infiltration into host epithelial cells (177). This interaction provides a long-term disabling effect and possibly even structural alteration of the bound toxin (188) which could prove useful in clinical application considering Stx, in conjunction to their ability to infiltrate host epithelial cells, also infiltrates underlying tissues (195).

9. Conclusions and Future Perspectives

I*n vitro* investigation of NDOs has unveiled a wide range of anti-pathogenic functionalities, including anti-adhesion properties against pathogens, inhibition of biofilm formation, inhibition of specific pathogen growth and toxin-binding properties. An overview of these anti-pathogenic functionalities with corresponding NDOs is illustrated in Figure 8.

Most of the anti-pathogenic functionalities elicited by a specific oligosaccharide can be predicted by investigation of a number of characteristics, for example, the presence of a pathogenic adhesin or toxin that may bind to the carbohydrate sequence and/or potential charge of the oligosaccharide. However, structural features of oligosaccharides responsible for their adhesin-specific anti-adhesion properties are not necessarily related to mechanisms of other anti-pathogenic activities. Ionic interaction between charged oligosaccharides and pathogenic exterior can cause decreased motility and transport of nutrition, while some NDOs may electrostatically interact with intracellular DNA, inhibiting DNA-transcription.

Although several mechanisms of anti-pathogenic functioning have been identified, not all studies propose a clear explanation for the observed anti-microbial properties, and there are also contradictory reports concerning the antimicrobial potential of several NDOs against different types of microbial strains. A better characterization of the oligosaccharides in terms of DP, DA and monosaccharide sequence and testing a wider range of pathogens could assist in further uncovering details of anti-pathogenic functionalities. Glycan (or carbohydrate) arrays (e.g., using glycan probes) could also contribute to fast and high-throughput screening of protein-carbohydrate interactions with small amounts of carbohydrate ligands (196,197).

For clinical application, monotherapy with single adhesin-specific NDOs will have limited chance of successfully inhibiting pathogen adhesion, due to the variability of pathogenic adhesin expression. Rather, a mixture of NDOs with affinity for different adhesins could

have a higher clinical applicability and in general, NDOs are not pure products, but are mixtures containing oligosaccharides of different DP.

For direct inhibition of pathogens, charged NDOs are more interesting to the clinical environment compared to uncharged NDOs due to lower pathogen-specificity, not relying on expression of a specific pathogenic adhesin. Non-food application, including local application via lung or skin, can be proposed for future investigation. Before we can make any statements about future applications requiring systemic delivery of NDOs, assessment of systemic stability, toxicity and immunogenicity of NDOs is needed.

In conclusion, versatility of antimicrobial effects, their unique ability to penetrate and inhibit biofilm structures and their limited side effects plea for oligosaccharides as a useful tool in the battle against emerging infections and antibiotic resistance. In addition, the effects of NDOs on promoting beneficial bacteria in the gut should not be neglected, since a well-balanced microbiota contributes to protection against infections by inhibiting pathogenic bacteria or by orchestrating appropriate immune responses.

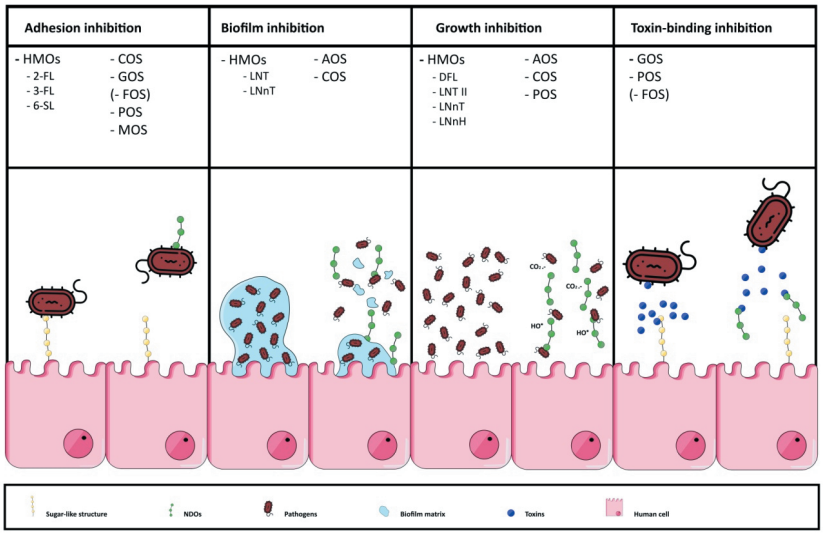

Figure 8. Schematic overview of the anti-pathogenic functionalities of NDOs *in vitro*. The first column shows that different NDOs (different HMOs, COS, GOS, FOS, POS and MOS) can serve as decoy receptors that competitively bind pathogens, which prevents pathogen adhesion to cell surface glycans. The second column indicates that several HMOs, AOS and COS can inhibit biofilm formation by penetrating and interacting with elements of the pathogenic biofilm. Multiple NDOs, such as, different HMOs, AOS, COS and POS, have shown to inhibit pathogenic growth, for example, by disrupting the bacterial cell membrane and/or by scavenging free radicals, such as HO•, which have a number of pathological effects (third column). The fourth column indicates that several NDOs (GOS, POS, FOS) can inhibit host cell interaction with pathogenic toxins.

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References

1. Mussatto SI, Mancilha IM. Non-digestible oligosaccharides: A review. Carbohydrate Polymers. 2007 Apr 5;68(3):587–97.
2. Akbari P, Fink-Gremmels J, Willems RHAM, Difilippo E, Schols HA, Schoterman MHC, et al. Characterizing microbiota-independent effects of oligosaccharides on intestinal epithelial cells: insight into the role of structure and size. European Journal of Nutrition [Internet]. 2017;56(5):1919–30. Available from: https://doi.org/10.1007/s00394-016-1234-9
3. Schouten B, van Esch BCAM, Kormelink TG, Moro GE, Arslanoglu S, Boehm G, et al. Non-digestible oligosaccharides reduce immunoglobulin free light-chain concentrations in infants at risk for allergy. Pediatric Allergy and Immunology. 2011;22(5):537–42.
4. Algieri F, Rodriguez-Nogales A, Garrido-Mesa N, Vezza T, Garrido-Mesa J, Utrilla MP, et al. Intestinal anti-inflammatory effects of oligosaccharides derived from lactulose in the trinitrobenzenesulfonic acid model of rat colitis. J Agric Food Chem. 2014;62(19):4285–97.
5. Okeke IN, Aboderin OA, Byarugaba DK, Ojo KK, Opintan JA. Growing problem of multidrug-resistant enteric pathogens in Africa. Emerg Infect Dis. 2007;13(11):1640.
6. Humphries RM, Schuetz AN. Antimicrobial susceptibility testing of bacteria that cause gastroenteritis. Clinics in Laboratory Medicine. 2015;35(2):313–31.
7. Willyard C. The drug-resistant bacteria that pose the greatest health threats. Nature News. 2017;543(7643):15.
8. Flemming HC, Wingender J. The biofilm matrix. Nature reviews microbiology. 2010;8(9):623–33.
9. Qiang X, YongLie C, QianBing W. Health benefit application of functional oligosaccharides. Carbohydr Polym. 2009;77(3):435–41.
10. Cai Y, Folkerts J, Folkerts G, Maurer M, Braber S. Microbiota-dependent and-independent effects of dietary fibre on human health. Br J Pharmacol. 2020;177(6):1363–81.
11. Haak BW, Prescott HC, Wiersinga WJ. Therapeutic potential of the gut microbiota in the prevention and treatment of sepsis. Front Immunol. 2018;2042.
12. Vitetta L, Vitetta G, Hall S. Immunological tolerance and function: associations between intestinal bacteria, probiotics, prebiotics, and phages. Front Immunol. 2018;2240.
13. Bernardi A, Jiménez-Barbero J, Casnati A, de Castro C, Darbre T, Fieschi F, et al. Multivalent glycoconjugates as anti-pathogenic agents. Chemical Society Reviews. 2013;42(11):4709–27.
14. Shoaf-Sweeney KD, Hutkins RW. Adherence, anti-adherence, and oligosaccharides: preventing pathogens from sticking to the host. Adv Food Nutr Res. 2008;55:101–61.
15. Rosenberg M, Bayer EA, Delarea J, Rosenberg E. Role of thin fimbriae in adherence and growth of Acinetobacter calcoaceticus RAG-1 on hexadecane. Applied and Environmental Microbiology. 1982;44(4):929–37.
16. Duguid JP, Old DC. Adhesive properties of Enterobacteriaceae. In: Bacterial adherence. Springer; 1980. p. 185–217.
17. Coppa G v, Zampini L, Galeazzi T, Facinelli B, Ferrante L, Capretti R, et al. Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: Escherichia coli, Vibrio cholerae, and Salmonella fyris. Pediatr Res. 2006;59(3):377–82.
18. Newburg DS. Do the binding properties of oligosaccharides in milk protect human infants from gastrointestinal bacteria? J Nutr. 1997;127(5):980S-984S.
19. Lin AE, Autran CA, Szyszka A, Escajadillo T, Huang M, Godula K, et al. Human milk oligosaccharides inhibit growth of group B *Streptococcus*. Journal of Biological Chemistry. 2017;292(27):11243–9.
20. Andreas NJ, Al-Khalidi A, Jaiteh M, Clarke E, Hyde MJ, Modi N, et al. Role of human milk oligosaccharides in Group B *Streptococcus* colonisation. Clin Transl Immunology. 2016;5(8):e99.
21. Ackerman DL, Doster RS, Weitkamp JH, Aronoff DM, Gaddy JA, Townsend SD. Human milk oligosaccharides exhibit antimicrobial and antibiofilm properties against group B *Streptococcus*. ACS Infect Dis. 2017;3(8):595–605.
22. Patel S, Goyal A. Functional oligosaccharides: production, properties and applications. World Journal of Microbiology and Biotechnology. 2011;27(5):1119–28.

23. Smilowitz JT, Lebrilla CB, Mills DA, German JB, Freeman SL. Breast milk oligosaccharides: structure-function relationships in the neonate. Annu Rev Nutr. 2014;34:143–69.
24. Wiciński M, Sawicka E, Gębalski J, Kubiak K, Malinowski B. Human milk oligosaccharides: Health benefits, potential applications in infant formulas, and pharmacology. Nutrients. 2020;12(1):266.
25. Bode L. Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology. 2012;22(9):1147–62.
26. Craft KM, Townsend SD. Mother knows best: deciphering the antibacterial properties of human milk oligosaccharides. Acc Chem Res. 2019;52(3):760–8.
27. Martín-Sosa S, Martín MJ, Hueso P. The sialylated fraction of milk oligosaccharides is partially responsible for binding to enterotoxigenic and uropathogenic Escherichia coli human strains. J Nutr. 2002;132(10):3067–72.
28. Coppa G v, Gabrielli O, Giorgi P, Catassi C, Montanari MP, Varaldo PE, et al. Preliminary study of breastfeeding and bacterial adhesion to uroepithelial cells. The Lancet. 1990;335(8689):569–71.
29. Andersson B, Porras O, Hanson LÅ, Lagergård T, Svanborg-Edén C. Inhibition of attachment of *Streptococcus* pneumoniae and Haemophilus influenzae by human milk and receptor oligosaccharides. Journal of infectious diseases. 1986;153(2):232–7.
30. Pham TAN, Clare S, Goulding D, Arasteh JM, Stares MD, Browne HP, et al. Epithelial IL-22RA1-mediated fucosylation promotes intestinal colonization resistance to an opportunistic pathogen. Cell Host Microbe. 2014;16(4):504–16.
31. Asahara T, Takahashi A, Yuki N, Kaji R, Takahashi T, Nomoto K. Protective effect of a synbiotic against multidrug-resistant Acinetobacter baumannii in a murine infection model. Antimicrob Agents Chemother. 2016;60(5):3041–50.
32. Goto Y, Obata T, Kunisawa J, Sato S, Ivanov II, Lamichhane A, et al. Innate lymphoid cells regulate intestinal epithelial cell glycosylation. Science (1979). 2014;345(6202):1254009.
33. Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. Annu Rev Nutr. 2005;25:37–58.
34. Cravioto A, Tello A, Villafan H, Ruiz J, del Vedovo S, Neeser JR. Inhibition of localized adhesion of enteropathogenic Escherichia coli to HEp-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk. Journal of Infectious Diseases. 1991;163(6):1247–55.
35. Hakkarainen J, Toivanen M, Leinonen A, Frängsmyr L, Strömberg N, Lapinjoki S, et al. Human and bovine milk oligosaccharides inhibit Neisseria meningitidis pili attachment *in vitro*. J Nutr. 2005;135(10):2445–8.
36. Kobata A. Structures and application of oligosaccharides in human milk. Proceedings of the Japan Academy, Series B. 2010;86(7):731–47.
37. Craft KM, Gaddy JA, Townsend SD. Human milk oligosaccharides (HMOs) sensitize group B *Streptococcus* to clindamycin, erythromycin, gentamicin, and minocycline on a strain specific basis. ACS Chemical Biology. 2018;13(8):2020–6.
38. Hsu Chen CC, Feingold DS. Mechanism of polymyxin B action and selectivity toward biologic membranes. Biochemistry. 1973;12(11):2105–11.
39. Lin AE, Autran CA, Espanola SD, Bode L, Nizet V. Human milk oligosaccharides protect bladder epithelial cells against uropathogenic Escherichia coli invasion and cytotoxicity. J Infect Dis. 2014;209(3):389–98.
40. Turner CE. Paxillin and focal adhesion signalling. Nat Cell Biol. 2000;2(12):E231–6.
41. Schumacher G, Bendas G, Stahl B, Beermann C. Human milk oligosaccharides affect P-selectin binding capacities: *in vitro* investigation. Nutrition. 2006;22(6):620–7.
42. Craft KM, Thomas HC, Townsend SD. Interrogation of human milk oligosaccharide fucosylation patterns for antimicrobial and antibiofilm trends in group B *streptococcus*. ACS Infectious Diseases. 2018;4(12):1755–65.
43. Ackerman DL, Craft KM, Doster RS, Weitkamp JH, Aronoff DM, Gaddy JA, et al. Antimicrobial and antibiofilm activity of human milk oligosaccharides against *Streptococcus* agalactiae, Staphylococcus aureus, and Acinetobacter baumannii. ACS Infect Dis. 2017;4(3):315–24.
44. Craft KM, Thomas HC, Townsend SD. Sialylated variants of lacto-N-tetraose exhibit antimicrobial activity against Group B *Streptococcus*. Organic & Biomolecular Chemistry. 2019;17(7):1893–900.

45. Bode L, Jantscher-Krenn E. Structure-function relationships of human milk oligosaccharides. Advances in Nutrition. 2012;3(3):383S-391S.
46. Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS. Campylobacter jejuni binds intestinal H (O) antigen (Fucα1, 2Galβ1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. Journal of biological chemistry. 2003;278(16):14112–20.
47. Weichert S, Jennewein S, Hüfner E, Weiss C, Borkowski J, Putze J, et al. Bioengineered 2′-fucosyllactose and 3-fucosyllactose inhibit the adhesion of Pseudomonas aeruginosa and enteric pathogens to human intestinal and respiratory cell lines. Nutrition research. 2013;33(10):831–8.
48. Obermeier S, Rudloff S, Pohlentz G, Lentze MJ, Kunz C. Secretion of 13C-labelled oligosaccharides into human milk and infant's urine after an oral 13C-galactose load. Isotopes in Environmental and Health Studies. 1999;35(1–2):119–25.
49. Craft KM, Townsend SD. 1-Amino-2′-fucosyllactose inhibits biofilm formation by *Streptococcus* agalactiae. The Journal of Antibiotics. 2019;72(6):507–12.
50. Thurl S, Munzert M, Boehm G, Matthews C, Stahl B. Systematic review of the concentrations of oligosaccharides in human milk. Nutrition Reviews. 2017;75(11):920–33.
51. Ninonuevo MR, Park Y, Yin H, Zhang J, Ward RE, Clowers BH, et al. A strategy for annotating the human milk glycome. J Agric Food Chem. 2006;54(20):7471–80.
52. Marotta M, Ryan JT, Hickey RM. The predominant milk oligosaccharide 6′-sialyllactose reduces the internalisation of Pseudomonas aeruginosa in human pneumocytes. J Funct Foods. 2014;6:367–73.
53. Lee JM, Oh SY, Johnston T v, Ku S, Ji GE. Biocatalysis of fucodian in Undaria pinnatifida sporophyll using Bifidobacterium longum RD47 for production of prebiotic fucosylated oligosaccharide. Marine Drugs. 2019;17(2):117.
54. Fitton JH. Therapies from fucoidan; multifunctional marine polymers. Mar Drugs. 2011;9(10):1731–60.
55. Hanisch FG, Hansman GS, Morozov V, Kunz C, Schroten H. Avidity of α-fucose on human milk oligosaccharides and blood group–unrelated oligo/polyfucoses is essential for potent norovirus-binding targets. Journal of Biological Chemistry. 2018;293(30):11955–65.
56. Newburg DS, Ruiz-Palacios GM, Altaye M, Chaturvedi P, Meinzen-Derr J, Guerrero M de L, et al. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. Glycobiology. 2004;14(3):253–63.
57. Rehm BHA. Bacterial polymers: biosynthesis, modifications and applications. Nature Reviews Microbiology. 2010;8(8):578–92.
58. Lattner D, Flemming HC, Mayer C. 13C-NMR study of the interaction of bacterial alginate with bivalent cations. Int J Biol Macromol. 2003;33(1–3):81–8.
59. Skj G, Grasdalen H, Larsen B. Monomer sequence and acetylation pattern in some bacterial alginates. Carbohydr Res. 1986;154(1):239–50.
60. Remminghorst U, Rehm BHA. Bacterial alginates: from biosynthesis to applications. Biotechnol Lett. 2006;28(21):1701–12.
61. Schürks N, Wingender J, Flemming HC, Mayer C. Monomer composition and sequence of alginates from Pseudomonas aeruginosa. Int J Biol Macromol. 2002;30(2):105–11.
62. Overhage J, Lewenza S, Marr AK, Hancock REW. Identification of genes involved in swarming motility using a Pseudomonas aeruginosa PAO1 mini-Tn 5-lux mutant library. J Bacteriol. 2007;189(5):2164–9.
63. Khan S, Tøndervik A, Sletta H, Klinkenberg G, Emanuel C, Onsøyen E, et al. Overcoming drug resistance with alginate oligosaccharides able to potentiate the action of selected antibiotics. Antimicrob Agents Chemother. 2012;56(10):5134–41.
64. Powell LC, Sowedan A, Khan S, Wright CJ, Hawkins K, Onsøyen E, et al. The effect of alginate oligosaccharides on the mechanical properties of Gram-negative biofilms. Biofouling. 2013;29(4):413–21.

65. Powell LC, Pritchard MF, Emanuel C, Onsøyen E, Rye PD, Wright CJ, et al. A nanoscale characterization of the interaction of a novel alginate oligomer with the cell surface and motility of Pseudomonas aeruginosa. Am J Respir Cell Mol Biol. 2014;50(3):483–92.
66. Pritchard MF, Powell LC, Jack AA, Powell K, Beck K, Florance H, et al. A low-molecular-weight alginate oligosaccharide disrupts pseudomonal microcolony formation and enhances antibiotic effectiveness. Antimicrob Agents Chemother. 2017;61(9):e00762-17.
67. Pritchard MF, Powell LC, Khan S, Griffiths PC, Mansour OT, Schweins R, et al. The antimicrobial effects of the alginate oligomer OligoG CF-5/20 are independent of direct bacterial cell membrane disruption. Sci Rep. 2017;7(1):1–12.
68. Overhage J, Bains M, Brazas MD, Hancock REW. Swarming of Pseudomonas aeruginosa is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. Am Soc Microbiol; 2008.
69. Powell LC, Pritchard MF, Ferguson EL, Powell KA, Patel SU, Rye PD, et al. Targeted disruption of the extracellular polymeric network of Pseudomonas aeruginosa biofilms by alginate oligosaccharides. npj Biofilms and Microbiomes. 2018;4(1):1–10.
70. Roberts JL, Khan S, Emanuel C, Powell LC, Pritchard MF, Onsøyen E, et al. An *in vitro* study of alginate oligomer therapies on oral biofilms. J Dent. 2013;41(10):892–9.
71. Körstgens V, Flemming HC, Wingender J, Borchard W. Influence of calcium ions on the mechanical properties of a model biofilm of mucoid Pseudomonas aeruginosa. Water science and technology. 2001;43(6):49–57.
72. Aslam SN, Newman MA, Erbs G, Morrissey KL, Chinchilla D, Boller T, et al. Bacterial polysaccharides suppress induced innate immunity by calcium chelation. Current Biology. 2008;18(14):1078–83.
73. Pier GB, Coleman F, Grout M, Franklin M, Ohman DE. Role of alginate O acetylation in resistance of mucoid Pseudomonas aeruginosa to opsonic phagocytosis. Infect Immun. 2001;69(3):1895–901.
74. Nivens DE, Ohman DE, Williams J, Franklin MJ. Role of alginate and its O acetylation in formation of Pseudomonas aeruginosa microcolonies and biofilms. J Bacteriol. 2001;183(3):1047–57.
75. Sarkisova S, Patrauchan MA, Berglund D, Nivens DE, Franklin MJ. Calcium-induced virulence factors associated with the extracellular matrix of mucoid Pseudomonas aeruginosa biofilms. J Bacteriol. 2005;187(13):4327–37.
76. Banin E, Vasil ML, Greenberg EP. Iron and Pseudomonas aeruginosa biofilm formation. Proceedings of the National Academy of Sciences. 2005;102(31):11076–81.
77. Wiens JR, Vasil AI, Schurr MJ, Vasil ML. Iron-regulated expression of alginate production, mucoid phenotype, and biofilm formation by Pseudomonas aeruginosa. MBio. 2014;5(1):e01010-13.
78. Falkeborg M, Cheong LZ, Gianfico C, Sztukiel KM, Kristensen K, Glasius M, et al. Alginate oligosaccharides: Enzymatic preparation and antioxidant property evaluation. Food Chem. 2014;164:185–94.
79. Jiang Q, Chen J, Yang C, Yin Y, Yao K. Quorum sensing: a prospective therapeutic target for bacterial diseases. BioMed Research International. 2019;2019.
80. Williams P, Cámara M. Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules. Curr Opin Microbiol. 2009;12(2):182–91.
81. Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, et al. A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms. Mol Microbiol. 2006;59(4):1114–28.
82. Yang L, Barken KB, Skindersoe ME, Christensen AB, Givskov M, Tolker-Nielsen T. Effects of iron on DNA release and biofilm development by Pseudomonas aeruginosa. Microbiology (N Y). 2007;153(5):1318–28.
83. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science (1979). 2002;295(5559):1487.
84. Jack AA, Khan S, Powell LC, Pritchard MF, Beck K, Sadh H, et al. Alginate oligosaccharide-induced modification of the lasI-lasR and rhlI-rhlR quorum-sensing systems in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2018;62(5):e02318-17.

85. Phillips PL, Schultz GS. Molecular mechanisms of biofilm infection: biofilm virulence factors. Advances in Wound Care. 2012;1(3):109–14.
86. Das T, Manefield M. Pyocyanin promotes extracellular DNA release in Pseudomonas aeruginosa. 2012;
87. Elkins JG, Hassett DJ, Stewart PS, Schweizer HP, McDermott TR. Protective role of catalase in Pseudomonas aeruginosa biofilm resistance to hydrogen peroxide. Appl Environ Microbiol. 1999;65(10):4594–600.
88. He X, Hwang H min, Aker WG, Wang P, Lin Y, Jiang X, et al. Synergistic combination of marine oligosaccharides and azithromycin against Pseudomonas aeruginosa. Microbiol Res. 2014;169(9–10):759–67.
89. Merz RA, Horsch M, Nyhlén LE, Rast DM. Jollès, P, Muzzarelli, RAA, Eds.; Chitin and Chitinases. Birkhäuser Verlag: Basel, Switzerland; 1999.
90. Liaqat F, Eltem R. Chitooligosaccharides and their biological activities: A comprehensive review. Carbohydr Polym. 2018;184:243–59.
91. Lodhi G, Kim YS, Hwang JW, Kim SK, Jeon YJ, Je JY, et al. Chitooligosaccharide and its derivatives: preparation and biological applications. Biomed Res Int. 2014;2014.
92. Mourya VK, Inamdar NN, Choudhari YM. Chitooligosaccharides: Synthesis, characterization and applications. Polymer Science Series A. 2011;53(7):583–612.
93. No HK, Park NY, Lee SH, Meyers SP. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. Int J Food Microbiol. 2002;74(1–2):65–72.
94. Zheng LY, Zhu JF. Study on antimicrobial activity of chitosan with different molecular weights. Carbohydr Polym. 2003;54(4):527–30.
95. Fernandes JC, Tavaria FK, Soares JC, Ramos ÓS, Monteiro MJ, Pintado ME, et al. Antimicrobial effects of chitosans and chitooligosaccharides, upon Staphylococcus aureus and Escherichia coli, in food model systems. Food Microbiology. 2008;25(7):922–8.
96. Kim SK, Rajapakse N. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. Carbohydr Polym. 2005;62(4):357–68.
97. Kurita K, Kaji Y, Mori T, Nishiyama Y. Enzymatic degradation of β-chitin: susceptibility and the influence of deacetylation. Carbohydrate Polymers. 2000;42(1):19–21.
98. Riegman N, Kusters R, van Veggel H, Bergmans H, van Bergen en Henegouwen P, Hacker J, et al. F1C fimbriae of a uropathogenic Escherichia coli strain: genetic and functional organization of the foc gene cluster and identification of minor subunits. J Bacteriol. 1990;172(2):1114–20.
99. Kim SK, Park PJ, Yang HP, Han SS. Subacute toxicity of chitosan oligosaccharide in Sprague-Dawley rats. Arzneimittelforschung. 2001;51(09):769–74.
100. VandeVord PJ, Matthew HWT, DeSilva SP, Mayton L, Wu B, Wooley PH. Evaluation of the biocompatibility of a chitosan scaffold in mice. Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials. 2002;59(3):585–90.
101. Allan CR, Hadwiger LA. The fungicidal effect of chitosan on fungi of varying cell wall composition. Exp Mycol. 1979;3(3):285–7.
102. Choi BK, Kim KY, Yoo YJ, Oh SJ, Choi JH, Kim CY. *In vitro* antimicrobial activity of a chitooligosaccharide mixture against Actinobacillus actinomycetemcomitans and *Streptococcus* mutans. Int J Antimicrob Agents. 2001;18(6):553–7.
103. Sánchez Á, Mengíbar M, Rivera-Rodríguez G, Moerchbacher B, Acosta N, Heras A. The effect of preparation processes on the physicochemical characteristics and antibacterial activity of chitooligosaccharides. Carbohydr Polym. 2017;157:251–7.
104. Fernandes JC, Tavaria FK, Fonseca SC, Ramos ÓS, Pintado ME, Malcata FX. *In vitro* screening for antimicrobial activity of chitosans and chitooligosaccharides, aiming at potential uses in functional textiles. J Microbiol Biotechnol. 2010;20(2):311–8.
105. Jeon YJ, Park PJ, Kim SK. Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr Polym. 2001;44(1):71–6.
106. Helander IM, Latva-Kala K, Lounatmaa K. Permeabilizing action of polyethyleneimine on Salmonella typhimurium involves disruption of the outer membrane and interactions with lipopolysaccharide. Microbiology (N Y). 1998;144(2):385–90.

107. Demeneix B, Behr J. Polyethylenimine (PEI). Adv Genet. 2005;53:215–30.
108. Vancha AR, Govindaraju S, Parsa KVL, Jasti M, González-García M, Ballestero RP. Use of polyethyleneimine polymer in cell culture as attachment factor and lipofection enhancer. BMC Biotechnol. 2004;4(1):1–12.
109. Helander IM, Nurmiaho-Lassila EL, Ahvenainen R, Rhoades J, Roller S. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int J Food Microbiol. 2001;71(2–3):235–44.
110. Young DH, Köhle H, Kauss H. Effect of chitosan on membrane permeability of suspension-cultured Glycine max and Phaseolus vulgaris cells. Plant Physiol. 1982;70(5):1449–54.
111. Young DH, Kauss H. Release of calcium from suspension-cultured Glycine max cells by chitosan, other polycations, and polyamines in relation to effects on membrane permeability. Plant Physiology. 1983;73(3):698–702.
112. Vishu Kumar AB, Varadaraj MC, Gowda LR, Tharanathan RN. Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of papain and Pronase, and their bactericidal action against Bacillus cereus and Escherichia coli. Biochemical Journal. 2005;391(2):167–75.
113. Moon JS, Kim HK, Koo HC, Joo YS, Nam H mi, Park YH, et al. The antibacterial and immunostimulative effect of chitosan-oligosaccharides against infection by Staphylococcus aureus isolated from bovine mastitis. Appl Microbiol Biotechnol. 2007;75(5):989–98.
114. Benhabiles MS, Salah R, Lounici H, Drouiche N, Goosen MFA, Mameri N. Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. Food Hydrocoll. 2012;29(1):48–56.
115. Quintero-Villegas MI, Aam BB, Rupnow J, Sørlie M, Eijsink VGH, Hutkins RW. Adherence inhibition of enteropathogenic Escherichia coli by chitooligosaccharides with specific degrees of acetylation and polymerization. J Agric Food Chem. 2013;61(11):2748–54.
116. Rhoades J, Gibson G, Formentin K, Beer M, Rastall R. Inhibition of the adhesion of enteropathogenic Escherichia coli strains to HT-29 cells in culture by chito-oligosaccharides. Carbohydr Polym. 2006;64(1):57–9.
117. Hadwiger LA, Beckman JM. Chitosan as a component of pea-Fusarium solani interactions. Plant Physiology. 1980;66(2):205–11.
118. LIU XF, Feng XQ, Yang S, Wang TP, SU ZHX. Effects of molecularweight and concentration of chitosan on antifungal activity against Aspergillus niger. 2008;
119. Sebti I, Martial-Gros A, Carnet-Pantiez A, Grelier S, Coma V. Chitosan polymer as bioactive coating and film against Aspergillus niger contamination. Journal of Food Science. 2005;70(2):M100–4.
120. Wagoner W, Loschke DC, Hadwiger LA. Two-dimensional electrophoretic analysis of in vivo and *in vitro* synthesis of proteins in peas inoculated with compatible and incompatible Fusarium solani. Physiological Plant Pathology. 1982;20(1):99–107.
121. Fei Liu X, Lin Guan Y, Zhi Yang D, Li Z, de Yao K. Antibacterial action of chitosan and carboxymethylated chitosan. J Appl Polym Sci. 2001;79(7):1324–35.
122. Raafat D, Sahl H. Chitosan and its antimicrobial potential–a critical literature survey. Microb Biotechnol. 2009;2(2):186–201.
123. Nikaido H. Multidrug resistance in bacteria. Annu Rev Biochem. 2009;78:119–46.
124. Tin S, Lim CS, Sakharkar MK, Sakharkar KR. Synergistic combinations of chitosans and antibiotics in Staphylococcus aureus. Letters in Drug Design & Discovery. 2010;7(1):31–5.
125. Tin S, Sakharkar KR, Lim CS, Sakharkar MK. Activity of Chitosans in combination with antibiotics in Pseudomonas aeruginosa. Int J Biol Sci. 2009;5(2):153.
126. Jeon YJ, Kim SK. Production of chitooligosaccharides using an ultrafiltration membrane reactor and their antibacterial activity. Carbohydr Polym. 2000;41(2):133–41.
127. Lee BC, Kim MS, Choi SH, Kim KY, Kim TS. *In vitro* and *in vivo* antimicrobial activity of water-soluble chitosan oligosaccharides against Vibrio vulnificus. Int J Mol Med. 2009;24(3):327–33.
128. Laokuldilok T, Potivas T, Kanha N, Surawang S, Seesuriyachan P, Wangtueai S, et al. Physicochemical, antioxidant, and antimicrobial properties of chitooligosaccharides produced using three different enzyme treatments. Food Biosci. 2017;18:28–33.

129. Roberfroid MB. Functional foods: concepts and application to inulin and oligofructose. British Journal of Nutrition. 2002;87(S2):S139–43.
130. Al-Ghazzewi FH, Tester RF. Inhibition of the adhesion of Escherichia coli to human epithelial cells by carbohydrates. Bioactive Carbohydrates and Dietary Fibre. 2014;4(1):1–5.
131. Kim JK, Shin EC, Park HG. Fructooligosaccharides decreased the ability of probiotic Escherichia coli Nissle 1917 to adhere to co-cultures of human intestinal cell lines. J Korean Soc Appl Biol Chem. 2015;58(1):45–52.
132. Ortega-Gonzalez M, Sanchez de Medina F, Molina-Santiago C, Lopez-Posadas R, Pacheco D, Krell T, et al. Fructooligosacharides reduce Pseudomonas aeruginosa PAO1 pathogenicity through distinct mechanisms. PLoS One. 2014;9(1):e85772.
133. Intanon M, Arreola SL, Pham NH, Kneifel W, Haltrich D, Nguyen TH. Nature and biosynthesis of galacto-oligosaccharides related to oligosaccharides in human breast milk. FEMS Microbiol Lett. 2014;353(2):89–97.
134. Rastall RA. Functional oligosaccharides: application and manufacture. Annu Rev Food Sci Technol. 2010;1:305–39.
135. Coulier L, Timmermans J, Bas R, van den Dool R, Haaksman I, Klarenbeek B, et al. In-depth characterization of prebiotic galacto-oligosaccharides by a combination of analytical techniques. Journal of Agricultural and Food Chemistry. 2009;57(18):8488–95.
136. Petri WA, Smith RD, Schlesinger PH, Murphy CF, Ravdin JI. Isolation of the galactose-binding lectin that mediates the *in vitro* adherence of Entamoeba histolytica. J Clin Invest. 1987;80(5):1238–44.
137. Petri Jr WA, Haque R, Mann BJ. The bittersweet interface of parasite and host: lectin-carbohydrate interactions during human invasion by the parasite Entamoeba histolytica. Annual Reviews in Microbiology. 2002;56(1):39–64.
138. Shoaf K, Mulvey GL, Armstrong GD, Hutkins RW. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic Escherichia coli to tissue culture cells. Infect Immun. 2006;74(12):6920–8.
139. Searle LEJ, Cooley WA, Jones G, Nunez A, Crudgington B, Weyer U, et al. Purified galactooligosaccharide, derived from a mixture produced by the enzymic activity of Bifidobacterium bifidum, reduces Salmonellaenterica serovar Typhimurium adhesion and invasion *in vitro* and in vivo. J Med Microbiol. 2010;59(12):1428–39.
140. Kittana H, Quintero-Villegas MI, Bindels LB, Gomes-Neto JC, Schmaltz RJ, Munoz RRS, et al. Galactooligosaccharide supplementation provides protection against Citrobacter rodentium-induced colitis without limiting pathogen burden. Microbiology (N Y). 2018;164(2):154.
141. Ebersbach T, Andersen JB, Bergström A, Hutkins RW, Licht TR. Xylo-oligosaccharides inhibit pathogen adhesion to enterocytes *in vitro*. Res Microbiol. 2012;163(1):22–7.
142. Quintero M, Maldonado M, Perez-Munoz M, Jimenez R, Fangman T, Rupnow J, et al. Adherence inhibition of Cronobacter sakazakii to intestinal epithelial cells by prebiotic oligosaccharides. Curr Microbiol. 2011;62(5):1448–54.
143. Mange JP, Stephan R, Borel N, Wild P, Kim KS, Pospischil A, et al. Adhesive properties of Enterobacter sakazakii to human epithelial and brain microvascular endothelial cells. BMC Microbiol. 2006;6(1):1–10.
144. Muanprasat C, Chatsudthipong V. Cholera: pathophysiology and emerging therapeutic targets. Future Med Chem. 2013;5(7):781–98.
145. Lanne B, Schierbeck B, Ångström J. Binding of Cholera Toxin B-Subunits to Derivatives of the Natural Ganglioside Receptor, GM 1. The Journal of Biochemistry. 1999;126(1):226–34.
146. Wallenfels K, Malhotra OP. Galactosidases. In: Advances in carbohydrate chemistry. Elsevier; 1962. p. 239–98.
147. Holmgren J. Actions of cholera toxin and the prevention and treatment of cholera. Nature. 1981;292(5822):413–7.
148. Haksar D, van Ufford LQ, Pieters RJ. A hybrid polymer to target blood group dependence of cholera toxin. Organic & Biomolecular Chemistry. 2020;18(1):52–5.

149. Sinclair HR, de Slegte J, Gibson GR, Rastall RA. Galactooligosaccharides (GOS) inhibit Vibrio cholerae toxin binding to its GM1 receptor. Journal of Agricultural and Food Chemistry. 2009;57(8):3113–9.
150. Zomer-van Ommen DD, Pukin A v, Fu O, Quarles Van Ufford LHC, Janssens HM, Beekman JM, et al. Functional characterization of cholera toxin inhibitors using human intestinal organoids. Journal of Medicinal Chemistry. 2016;59(14):6968–72.
151. Ofek I, Beachey EH. Mannose binding and epithelial cell adherence of Escherichia coli. Infection and Immunity. 1978;22(1):247–54.
152. Ofek I, Mirelman D, Sharon N. Adherence of Escherichia coli to human mucosal cells mediated by mannose receptors. Nature. 1977;265(5595):623–5.
153. Firon N, Ofek I, Sharon N. Carbohydrate specificity of the surface lectins of Escherichia coli, Klebsiella pneumoniae, and Salmonella typhimurium. Carbohydr Res. 1983;120:235–49.
154. Liu XY, Wang Q, Cui SW, Liu HZ. A new isolation method of β-D-glucans from spent yeast Saccharomyces cerevisiae. Food Hydrocolloids. 2008;22(2):239–47.
155. Jones CH, Pinkner JS, Roth R, Heuser J, Nicholes A v, Abraham SN, et al. FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the Enterobacteriaceae. Proceedings of the National Academy of Sciences. 1995;92(6):2081–5.
156. Krogfelt KA, Bergmans H, Klemm P. Direct evidence that the FimH protein is the mannose-specific adhesin of Escherichia coli type 1 fimbriae. Infect Immun. 1990;58(6):1995–8.
157. Firon N, Ofek I, Sharon N. Carbohydrate-binding sites of the mannose-specific fimbrial lectins of enterobacteria. Infect Immun. 1984;43(3):1088–90.
158. Firon N, Ashkenazi S, Mirelman D, Ofek I, Sharon N. Aromatic alpha-glycosides of mannose are powerful inhibitors of the adherence of type 1 fimbriated Escherichia coli to yeast and intestinal epithelial cells. Infect Immun. 1987;55(2):472–6.
159. Lindhorst TK, Kieburg C, Krallmann-Wenzel U. Inhibition of the type 1 fimbriae-mediated adhesion of Escherichia coli to erythrocytes by multiantennary [alpha]-mannosyl clusters: The effect of multivalency. Glycoconj J. 1998;15(6):605.
160. Bouckaert J, Mackenzie J, de Paz JL, Chipwaza B, Choudhury D, Zavialov A, et al. The affinity of the FimH fimbrial adhesin is receptor-driven and quasi-independent of Escherichia coli pathotypes. Mol Microbiol. 2006;61(6):1556–68.
161. Rosen DA, Pinkner JS, Walker JN, Elam JS, Jones JM, Hultgren SJ. Molecular variations in Klebsiella pneumoniae and Escherichia coli FimH affect function and pathogenesis in the urinary tract. Infect Immun. 2008;76(7):3346–56.
162. Sokurenko E v, Chesnokova V, Dykhuizen DE, Ofek I, Wu XR, Krogfelt KA, et al. Pathogenic adaptation of Escherichia coli by natural variation of the FimH adhesin. Proceedings of the National Academy of Sciences. 1998;95(15):8922–6.
163. Ramirez-Hernandez A, Rupnow J, Hutkins RW. Adherence reduction of Campylobacter jejuni and Campylobacter coli strains to HEp-2 cells by mannan oligosaccharides and a high-molecular-weight component of cranberry extract. J Food Prot. 2015;78(8):1496–505.
164. Ganner A, Schatzmayr G. Capability of yeast derivatives to adhere enteropathogenic bacteria and to modulate cells of the innate immune system. Appl Microbiol Biotechnol. 2012;95(2):289–97.
165. Ganner A, Stoiber C, Uhlik JT, Dohnal I, Schatzmayr G. Quantitative evaluation of *E. coli* F4 and Salmonella Typhimurium binding capacity of yeast derivatives. AMB Express. 2013;3(1):1–7.
166. Xu X, Qiao Y, Peng Q, Gao L, Shi B. Inhibitory effects of YCW and MOS from Saccharomyces cerevisiae on Escherichia coli and Salmonella pullorum adhesion to Caco-2 cells. Front Biol (Beijing). 2017;12(5):370–5.
167. Firon N, Ofek I, Sharon N. Interaction of mannose-containing oligosaccharides with the fimbrial lectin of Escherichia coli. Biochem Biophys Res Commun. 1982;105(4):1426–32.
168. Smith S, Elbein AD, Pan YT. Inhibition of bacterial binding by high-mannose oligosaccharides. Google Patents; 1999.
169. Mualikrishna G, Tharanathan RN. Characterization of pectic polysaccharides from pulse husks. Food Chem. 1994;50(1):87–9.

170. Thakur BR, Singh RK, Handa AK, Rao MA. Chemistry and uses of pectin—a review. Critical Reviews in Food Science & Nutrition. 1997;37(1):47–73.
171. May CD. Industrial pectins: sources, production and applications. Carbohydr Polym. 1990;12(1):79–99.
172. Morris GA, Foster TJ, Harding SE. The effect of the degree of esterification on the hydrodynamic properties of citrus pectin. Food Hydrocolloids. 2000;14(3):227–35.
173. Mandalari G, Bennett RN, Kirby AR, lo Curto RB, Bisignano G, Waldron KW, et al. Enzymatic hydrolysis of flavonoids and pectic oligosaccharides from bergamot (Citrus bergamia Risso) peel. J Agric Food Chem. 2006;54(21):8307–13.
174. Olano-Martin E, Williams MR, Gibson GR, Rastall RA. Pectins and pectic-oligosaccharides inhibit Escherichia coli O157: H7 Shiga toxin as directed towards the human colonic cell line HT29. FEMS Microbiol Lett. 2003;218(1):101–5.
175. Nangia-Makker P, Conklin J, Hogan V, Raz A. Carbohydrate-binding proteins in cancer, and their ligands as therapeutic agents. Trends Mol Med. 2002;8(4):187–92.
176. Li P jun, Xia J lan, Nie Z yuan, Shan Y. Pectic oligosaccharides hydrolyzed from orange peel by fungal multi-enzyme complexes and their prebiotic and antibacterial potentials. LWT-Food Science and Technology. 2016;69:203–10.
177. Ganan M, Collins M, Rastall R, Hotchkiss AT, Chau HK, Carrascosa A v, et al. Inhibition by pectic oligosaccharides of the invasion of undifferentiated and differentiated Caco-2 cells by Campylobacter jejuni. Int J Food Microbiol. 2010;137(2–3):181–5.
178. Li S, Li T, Zhu R, Wang N, Song Y, Wang S, et al. Antibacterial action of haw pectic oligosaccharides. Int J Food Prop. 2013;16(3):706–12.
179. Pristov JB, Jovanović SV, Mitrović A, Spasojević I. UV-irradiation provokes generation of superoxide on cell wall polygalacturonic acid. Physiol Plant. 2013;148(4):574–81.
180. Tazawa K. Inhibitory Effects of Apple Pectin in Colon Carcinogenesis-Evaluation of the Radical Scavenging Activity of Pectic Oligosaccharide. BIOTHERAPY-TOKYO-. 1999;13:510–2.
181. Marx JL. Oxygen Free Radicals Linked to Many Diseases: The oxygen free radicals, although made as by-products of normal oxygen-using reactions, nevertheless have a wide potential for causing cell injury. Science (1979). 1987;235(4788):529–31.
182. Zheng RL, Lesko SA, Ts'o PO. DNA damage induced in mammalian cells by active oxygen species. Scientia Sinica Series B, Chemical, Biological, Agricultural, Medical & Earth Sciences. 1988;31(6):676–86.
183. Slemmer JE, Shacka JJ, Sweeney MI, Weber JT. Antioxidants and free radical scavengers for the treatment of stroke, traumatic brain injury and aging. Curr Med Chem. 2008;15(4):404–14.
184. Martinov J, Krstić M, Spasić S, Miletić S, Stefanović-Kojić J, Nikolić-Kokić A, et al. Apple pectin-derived oligosaccharides produce carbon dioxide radical anion in Fenton reaction and prevent growth of Escherichia coli and Staphylococcus aureus. Food Research International. 2017;100:132–6.
185. Olano-Martin E, Mountzouris KC, Gibson GR, Rastall RA. Continuous production of pectic oligosaccharides in an enzyme membrane reactor. J Food Sci. 2001;66(7):966–71.
186. Rhoades J, Manderson K, Wells A, Hotchkiss Jr AT, Gibson GR, Formentin K, et al. Oligosaccharide-mediated inhibition of the adhesion of pathogenic Escherichia coli strains to human gut epithelial cells in vitro. J Food Prot. 2008;71(11):2272–7.
187. Guggenbichler JP, de Bettignies-Dutz A, Meissner P, Schellmoser S, Jurenitsch J. Acidic oligosaccharides from natural sources block adherence of Escherichia coli on uroepithelial cells. Pharmaceutical and Pharmacological Letters. 1997;7(1):35–8.
188. Di R, Vakkalanka MS, Onumpai C, Chau HK, White A, Rastall RA, et al. Pectic oligosaccharide structure-function relationships: Prebiotics, inhibitors of Escherichia coli O157: H7 adhesion and reduction of Shiga toxin cytotoxicity in HT29 cells. Food Chemistry. 2017;227:245–54.
189. Lee JH, Shim JS, Lee JS, Kim MK, Chung MS, Kim KH. Pectin-like acidic polysaccharide from Panax ginseng with selective antiadhesive activity against pathogenic bacteria. Carbohydrate Research. 2006;341(9):1154–63.

190. Cohen A, Hannigan GE, Williams BR, Lingwood CA. Roles of globotriosyl-and galabiosylceramide in verotoxin binding and high affinity interferon receptor. Journal of Biological Chemistry. 1987;262(35):17088–91.
191. Lingwood CA, Law H, Richardson S, Petric M, Brunton JL, de Grandis S, et al. Glycolipid binding of purified and recombinant Escherichia coli produced verotoxin *in vitro*. Journal of Biological Chemistry. 1987;262(18):8834–9.
192. Bergan J, Lingelem ABD, Simm R, Skotland T, Sandvig K. Shiga toxins. Toxicon. 2012;60(6):1085–107.
193. Melton-Celsa AR. Shiga toxin (Stx) classification, structure, and function. Microbiol Spectr. 2014;2(4):2–4.
194. Keusch GT, Jacewicz M, Mobassaleh M, Donohue-Rolfe A. Shiga toxin: intestinal cell receptors and pathophysiology of enterotoxic effects. Rev Infect Dis. 1991;13(Supplement_4):S304–10.
195. Acheson DW, Moore R, de Breucker S, Lincicome L, Jacewicz M, Skutelsky E, et al. Translocation of Shiga toxin across polarized intestinal cells in tissue culture. Infect Immun. 1996;64(8):3294–300.
196. Li Z, Feizi T. The neoglycolipid (NGL) technology-based microarrays and future prospects. FEBS Lett. 2018;592(23):3976–91.
197. Li Z, Chai W. Mucin O-glycan microarrays. Current opinion in Structural Biology. 2019;56:187–97.

Chapter 3

Differential effects of oligosaccharides on the effectiveness of ampicillin against *Escherichia coli in vitro*

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Abstract

The mounting antibiotic resistance emphasizes an urgent need for alternatives. Recent investigations indicate that non-digestible oligosaccharides (NDOs), besides their prebiotic properties, can directly interact with pathogenic bacteria. In this study, the protective effect of alginate-oligosaccharides (AOS), chitosan-oligosaccharides (COS), galacto-oligosaccharides (GOS) and fructo-oligosaccharides, against enteropathogenic *Escherichia. coli* was investigated. The effect of these NDOs on *E. coli* growth, adhesion and *E. coli*-induced inflammatory response (IL-8 release) of HT-29 intestinal epithelial cells was determined *in vitro* in the presence or absence of ampicillin, using minimum inhibitory concentration (MIC) assay, anti-adhesion assay and ELISA, respectively. At low concentrations 0.5% and 1% , AOS decreased the *E. coli* growth, while high GOS concentrations (6%, 8%, 10%) were effective. Interestingly, the combination of the low concentrations of AOS with ampicillin (2 μg/mL) exerted a 2-fold decrease in the MIC level of ampicillin against *E. coli*. AOS also concentration dependently reduced the adherence of *E. coli* to HT-29 cells. The combination of AOS with ampicillin further increased these anti-adhesive properties. Pre-incubation of HT-29 cells with AOS, COS or GOS significantly hampered the *E. coli*-induced IL-8 release. Current study highlights the direct effects of NDOs on *E. coli* growth, adhesion and inflammatory responses of HT-29 cells *in vitro*.

Keywords: Oligosaccharides, Ampicillin, *E. coli* , Bacterial growth, Anti-adhesion, Anti-inflammation

1. Introduction

Antibiotic resistance as one of global threats, is responsible for increased morbidity and mortality from antibiotic-resistant infections leading to an enormous increase in health-care costs (1). Examples of major mechanisms of bacterial resistance to antibiotics are biofilm formation, chromosomal mutations and horizontal gene transfer (2,3). These drug resistance mechanisms allow bacteria to survive, or even grow in the presence of an antibiotic, while certain bacterial strains develop resistance against multiple drugs (4). Moreover, the antibiotic use can alter the composition and balance of the human gastrointestinal microbiota resulting in dysbiosis (5), which can promote the colonization of (drug-resistant) pathogens. Following ongoing concerns about increasing prevalence of antibiotic resistance with a lack of investment in new antibiotic development and discovery (6), alternative strategies for antibiotic therapy are an obvious necessity to strengthen the effectivity of conventional antibiotics and decreasing unwanted side effects. In recent years a particular attention was paid to a balanced and healthy microbiota in defending humans from being colonized and infected by pathogenic bacteria, such as certain *Escherichia coli* (*E. coli*) variants (7). Non-digestible oligosaccharides (NDOs) are ingredients incorporated into foods, beverages and supplements, which may be called functional foods. These foods induce changes in the composition and/or the balance of the gastrointestinal microbiota; stimulating gut-health and promoting bacteria such as *Bifidobacterium*; leading to reduced colonization of pathogenic bacteria, inhibition of bacterial infections and stimulation of immune homeostasis (8–10). However, the beneficial effects of NDOs may go beyond microbiota manipulations, since there are indications that NDOs can directly interact with pathogenic bacteria (11). A unique antibacterial role by inhibiting pathogen growth (e.g. *Staphylococcus aureus*, *E. coli*) has been described for several NDOs, such as alginate-oligosaccharides (AOS) and chitosan oligosaccharides (COS) (12–14) These oligosaccharides display biofilm-inhibiting properties against different types of bacteria, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (15–17). In addition, NDOs inhibit colonization and attachment of specific pathogens. For example, the potent anti-adhesion activity of galacto-oligosaccharides (GOS) and COS against different pathogenic strains, such as *E. coli*, *Salmonella* serotype (18) and *Cronobacter sakazakii*, has been previously demonstrated (19–24). Recent *in vitro* investigations from our group and others showed that NDOs can even directly interact with immune and epithelial cells stimulating intestinal homeostasis (25–27). However, there is scarce information whether these NDOs can increase the effectiveness of selected antibiotics and reduce the antibiotic dose (15,17). Therefore, the present *in vitro* study aims to investigate the effects of different oligosaccharides and oligosaccharide concentrations on: 1) enteropathogenic bacterial growth and adhesion, 2) the release of inflammatory mediators from HT-29 intestinal epithelial cells and 3) the 'moderately effective' concentration of beta-lactam antibiotic (ampicillin) to suppress *E. coli* growth. Structurally different NDOs from various sources are used, including AOS, COS, GOS and fructo-oligosaccharides (FOS), with or without the

combination of ampicillin, to examine the effect on *E. coli* growth, *E. coli* attachment and inflammatory responses induced by *E. coli* using HT-29 intestinal epithelial cells.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

E. coli was obtained from American Type Tissue Culture Collection (ATCC-8739). Stock cultures of the bacterial strain were stored at -80 °C in Luria–Bertani (LB) broth supplemented with 15% (v/v) glycerol. Bacteria were seeded and grown overnight on sheep blood agar plates (bio TRADING, Mijdrecht, Netherlands) under aerobic conditions at 37°C without shaking. Single colonies were harvested from the blood agar plates and grown in tryptic soy broth (TSB) for 120-180 minutes to reach an optical density (OD) of 0.5 based on McFarland standard (equal to 4 $x10^8$ CFU/mL of *E. coli*).

2.2. Cell culture

Human colorectal adenocarcinoma HT-29 cell were obtained from ATCC (HTB-38). Cells were cultured in 75 cm^2 culture flasks in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 25 mM Hepes, 4.5 g/l glucose (Gibco, Invitrogen, Carlsbad, CA, USA), 10% (v/v) inactivated fetal calf serum (FCS) (Gibco), glutamine (2 mM, Biocambrex, Verviers, Belgium), 1% (v/v) nonessential amino acids, penicillin (100 U/mL) and streptomycin (100 g/mL) (Biocambrex) in a humidified atmosphere containing 5% CO_2/95% air at 37 °C. Confluent cells were trypsinized using 0.05% trypsin containing 0.54 mM ethylene diamine tetra-acetic acid (EDTA). For all experiments, cells were seeded at a density of 1.5 x 10^5/well in 24-well plates and were grown for 72 h (37 °C, 5% CO_2) till a confluent monolayer was achieved. The medium was refreshed every other day.

2.3. Oligosaccharides

Alginate oligosaccharides (AOS) produced by degradation of algin (purity >85%) and chitosan oligosaccharide (COS) derived from rich marine biological sources (shrimp & crab shells)(purity >90%) were purchased from BZ Oligo Biotech Co., Ltd. (Qingdao, Shandong, China). Fructo-oligosaccharides (FOS) (purity of >97%) isolated from chicory were obtained from Orafti (Wijchen, The Netherlands). Galacto-oligosaccharides (GOS) (Vivinal® GOS Powder, purity >70%) prepared from lactose were provided by FrieslandCampina (Amersfoort, The Netherlands). The stock solutions of all oligosaccharides were freshly prepared by dissolving the oligosaccharides in TSB (Minimum inhibitory concentration; MIC assay), phosphate-buffered saline (PBS) (adhesion assay) or DMEM (immune assay). In Fig. 1 the chemical structures of the different NDOs, AOS, COS, GOS and FOS are depicted.

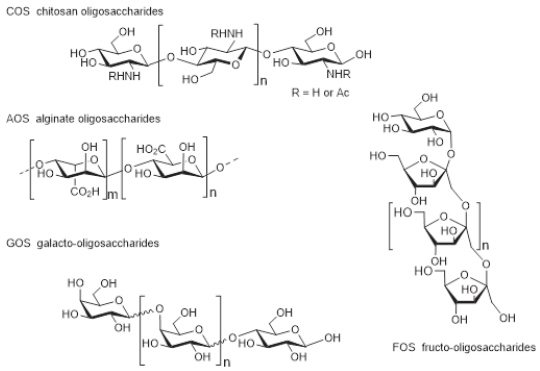

Figure 1. An overview of the chemical structures of the different NDOs (AOS, COS, FOS, GOS)

2.4. Antibiotic

Ampicillin was purchased from Sigma-Aldrich (Sigma-Aldrich, Steinheim, Germany) and freshly dissolved in TSB, PBS ,and DMEM prior to the MIC assay, adhesion assay and immune assay, respectively. From a preliminary MIC assay establishing the MIC for ampicillin (Fig. S1 A) the concentration of 0.2 µg/ml ampicillin (2 times lower than MIC) was selected for determining the differential effects of oligosaccharides on the effectiveness of ampicillin. For the adherence assay, in a preliminary assay (Fig. S1 B) the concentration of ampicillin with a moderate effectiveness (0.5 µg/mL) was selected in order to investigate the possible additive effects of oligosaccharides. Since the immune response induced by E. coli is in a direct relation with the adherence to the epithelial cells (28), the same concentration of ampicillin was used in the immune assay as well.

2.5. MIC (minimum inhibitory concentration)

The anti-bacterial capability of oligosaccharides in presence and absence of ampicillin was determined via analyzing the MIC following the method described by Qu *et al* (29). Different oligosaccharide concentrations (0.5% to 10%) and 2 µg/mL ampicillin (Fig. S1 A) were selected. A single E. coli colony was cultured in TSB medium to reach the optical density of 0.5 (OD: 0.5 = 4x108 CFU/mL) measured at 600 nm. To polypropylene round-bottom 96-well plates 25 µl from the serial dilutions of oligosaccharides with/without 25µl ampicillin and 50µl bacterial suspension were added. All plates were fully covered by sterile breathable film (VWR International, Amsterdam, Netherlands) and incubated overnight under shaking conditions (600 rpm). The MIC was considered when the wells did not show bacterial growth. Additionally, to quantify the observed MIC, the supernatants were gently transferred to a flat-bottom polystyrene 96-well plates and the OD was measured at 600 nm.

2.6. Anti-adhesion assay

The anti-adhesion assay was performed based on the protocol described by Wang *et al* (30). Briefly, culture media containing AOS, COS, GOS or FOS were prepared (0.25%-1%) in DMEM and were added to confluent HT29 monolayers in 24 well plates. After 24 h, the supernatants

were replaced with PBS containing oligosaccharides, ampicillin (0.5 µg/mL) and/or *E. coli* ($2x10^8$ CFU/mL). HT-29 cells were incubated for 2 h at 37 °C under aerobic conditions. Thereafter, cells were washed 3 times with PBS to discard non-adherent bacteria. Cells were lysed by 500 µL of 0.1% (v/v) Triton X-100 for 20 minutes at 37°C and cell lysates were cultured on blood agar. The bacterial adhesion was assessed by counting the number of the colonies after incubation in an aerobic incubator at 37°C for 15 h (Innova 4230 Shaker/Incubator (New Brunswick Scientific Co., Inc., Edison, NJ, USA). Data are presented as adhesive rate constant (ARC) (percentage of bacteria adhered relative to control).

2.7. Immune assay

Confluent HT-29 monolayers cultured in 24 well plates were pre-treated with oligosaccharides (0.25%-1%) for 24 h. Thereafter, the supernatants were replaced by DMEM medium containing oligosaccharides (0.25%-1%), the bacteria ($2x10^8$ CFU/mL (and/or ampicillin (0.5 µg/mL). After 4 h the culture supernatants were collected to measure IL-8 release by using IL-8 ELISA kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions.

2.8. Cell viability assay

Cell viability was examined using a 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) colorimetric assay (Sigma-Aldrich, St. Louis, Mo, USA). Briefly, HT-29 cells were grown on 24 well-plates for 72 h. The confluent monolayers were exposed to four different concentrations of oligosaccharides (0.25%, 0.5%, 1% and 2%), $2x10^8$ CFU/mL of bacteria and/or 2 µg/mL of ampicillin. MTT working solution (40 µl, 5 mg/mL in PBS) was added to the culture medium. After 2 h of incubation, the medium was removed, cells were lysed by DMSO and the absorbance was measured at 595 nm using iMark microplate reader (BioRad). The viability of the HT-29 cells was calculated based on the following equation: (mean absorbance of treatment cells / mean absorbance of control cells)*100

2.9. Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 8.0) (GraphPad, San Diego, CA, USA). Results are represented as mean values ± SEM of three independent experiments (n=3), each performed in triplicate (3 wells/condition). Differences between groups were statistically determined by using one way ANOVA with Bonferroni post-hoc test. The results were considered statistical significant when $P < 0.05$.

3. Results

3.1.1 Neither NDOs nor ampicillin or *E. coli* exert cytotoxic effects on HT-29 cells

Pre-incubation of HT-29 cells with AOS, COS, FOS and GOS for 24 h did not exert any cytotoxic effect in concentrations up to 1% (Fig. S2 A-D), while 2% AOS, COS and GOS (except FOS) significantly reduced the cell viability (Fig. S2 A-D). No noticeable changes in HT-29 cell viability were detected till 4 h incubation with 2 x 10^8 CFU/mL *E. coli* (data not

shown). Furthermore, pre-incubation with oligosaccharides (0.25%, 0.5%, 1%) for 24 h in combination with *E. coli* ($2x10^8$ CFU/ mL) ± ampicillin (0.5 µg/ mL) for 4 h did not impair HT-29 cell viability (Fig. S3 A-H).

3.1.2 NDOs from various sources with and without ampicillin differentially affect *E. coli* growth.

A significant reduction in the bacterial density was observed using low AOS concentrations (0.5% and 1%) as compared to the control, while the higher concentrations of AOS (2-10%) did not affect bacterial density (Fig. 2 A). GOS significantly decreased the *E. coli* growth in the 3 highest concentrations (6%, 8% and 10%) compared to control (Fig. 2 D). Similar to the effects of GOS, FOS also decreased the bacterial growth, although this was not significant (Fig. 2 C). Unlike other oligosaccharides, COS significantly increased the *E. coli* growth (Fig. 2 B). In order to investigate the additive effect of oligosaccharides on ampicillin, combinations were tested. As shown in Fig. S1 A, the MIC of ampicillin against *E. coli* was 4 µg/mL and the sub-MIC ampicillin concentration, 2 µg/mL, was used for further analyses. Ampicillin supplementation to COS, GOS or FOS had no effect on bacterial growth, but even partially hampered the effect of ampicillin (Fig. 2 B-D). However, the combination of AOS (0.5% and 1%) and ampicillin (2 µg/mL) exerted a complete inhibition on *E. coli* growth (Fig. 2 A). The combination of 0.5% and 1% AOS with ampicillin displayed a 2-fold decrease in MIC compared to ampicillin.

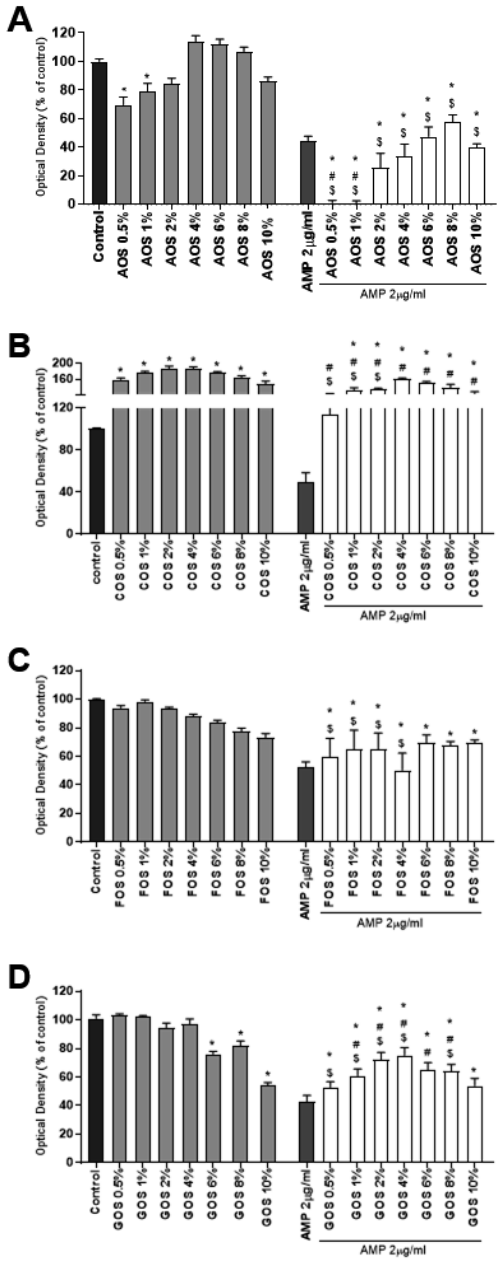

Figure 2. NDOs with and without ampicillin differentially affect *E. coli* growth. *E. coli* was grown overnight in presence or absence of AOS (a), COS (b), FOS (c) and GOS (d), with or without ampicillin and OD measurements (MIC assay) were used for determination of *E. coli* growth. Results are expressed as relative bacterial growth as mean ± SEM of three independent experiments each performed in triplicate. * = P<0.05 compared to control. # = P<0.05 compared to ampicillin. $= P<0.05 compared to corresponding concentration of oligosaccharides. Abbreviations: AMP, ampicillin; AOS, alginate oligosaccharides; COS, chitosan oligosaccharide; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.

3.1.3 NDOs from various sources with and without ampicillin differentially influence the adhesion of *E. coli* to HT-29 cells.

As shown in Fig. 3 A, pretreatment with 0.25%, 0.5% and 1% of AOS reduced the adherence of *E. coli* to HT-29 cells in a concentration-dependent manner. The combination of AOS (0.25%, 0.5% and 1%) with ampicillin further decreased the adhesive properties (Fig. 3 A). COS, GOS and FOS did not significantly alter the adherence of *E. coli* to HT-29 cells (Fig. 3 B-D) with or without ampicillin (Fig. 3 B-D).

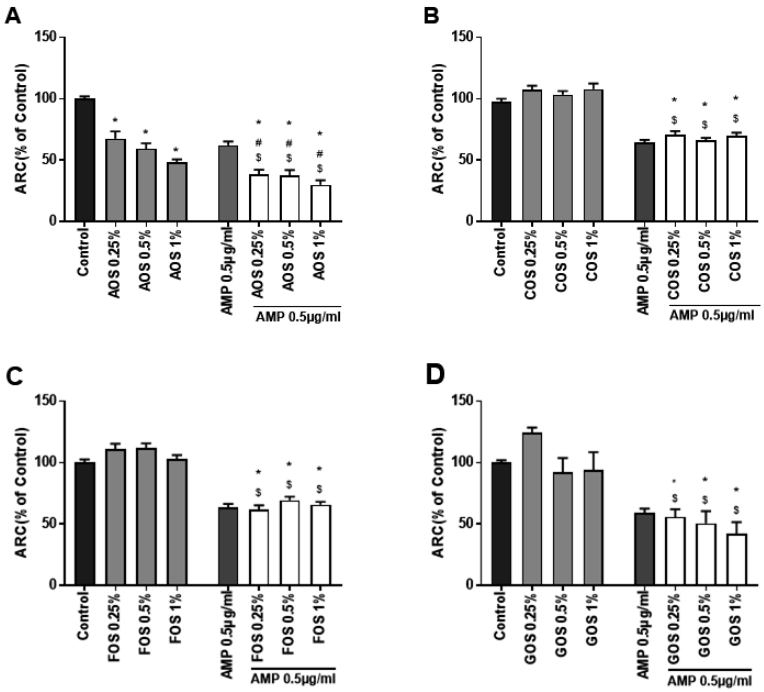

Figure 3. NDOs with and without ampicillin differentially influence the adhesion of *E. coli* to HT-29 cells. HT-29 cells, pre-treated in presence or absence of AOS (a), COS (b), FOS (c) and GOS (d) for 24 h, with or without ampicillin and exposed to *E. coli* for 2 h, were lysed and seeded on blood agar and colonies were counted. Results are expressed as adhesive rate constant (ARC) (percentage of bacteria adhered relative to control) as mean ± SEM of three independent experiments each performed in triplicate. * = P<0.05 compared to control. # = P<0.05 compared to ampicillin. $ = P<0.05 compared to corresponding concentration of oligosaccharides. Abbreviations: AMP, ampicillin; AOS, alginate oligosaccharides; COS, chitosan oligosaccharide; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.

3.1.4 NDOs from various sources reduce the *E. coli*-induced IL-8 release by HT-29 cells.

AOS, COS and GOS significantly decreased the *E. coli*-induced IL-8 release (Fig. 4A, B and D). Especially, the concentrations, 0.5% and 1% AOS and COS exerted a strong anti-inflammatory effect and these IL-8 levels were significantly less than in controls (Fig. 4 A and B). Pre-treatment of HT-29 cells with FOS did not result in modulation of *the E. coli*-induced IL-8 response in presence and absence of ampicillin. The combination of AOS,

COS, FOS, GOS with ampicillin, did not induce an additional effect on the *E. coli*-induced IL-8 release compared to the corresponding NDO concentrations.

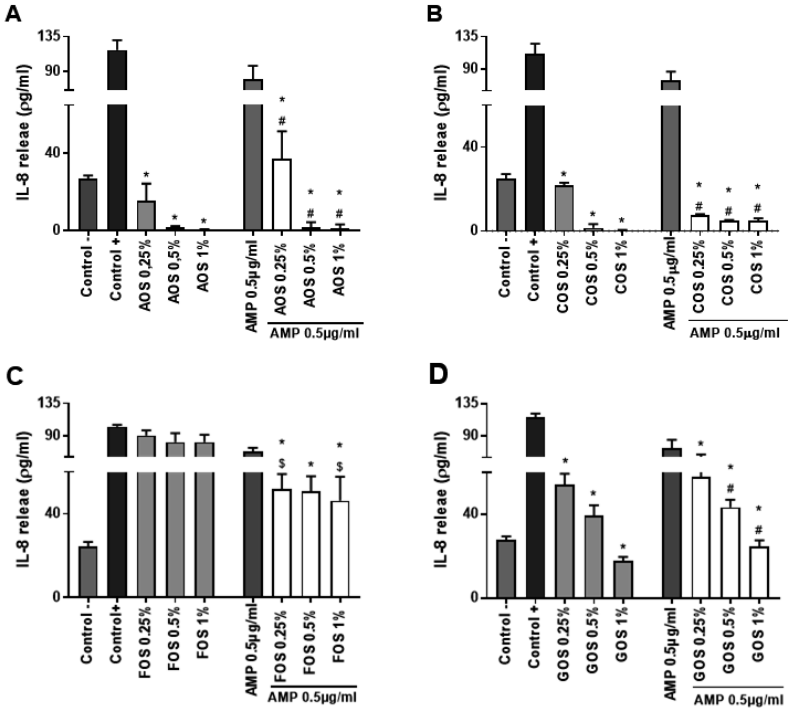

Figure 4. NDOs from various sources reduce the *E. coli*-induced IL-8 release by HT-29 cells. HT-29 cells, pre-treated with AOS (a), COS (b), FOS (c) and GOS (d), were exposed to *E. coli* in presence or absence of ampicillin (0.5 ug/mL) and IL-8 release was measured via ELISA. Results are expressed as relative IL-8 levels as mean ± SEM of three independent experiments each performed in triplicate. * = P<0.05 compared to control. # = P<0.05 compared to ampicillin. $ = P<0.05 compared to corresponding concentration of oligosaccharides. Abbreviations: AMP, ampicillin; AOS, alginate oligosaccharides; COS, chitosan oligosaccharide; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.

4. Discussion

This *in vitro* study aimed to investigate the effects of different oligosaccharides on enteropathogenic bacterial growth and adhesion, the release of inflammatory mediators from intestinal epithelial cells and the 'moderately effective' concentration of ampicillin to suppress *E. coli* growth. Differential effects of NDOs on *E. coli* growth were observed. It is generally known that *E. coli* can consume and grow on carbohydrates (31), which could be related to the observed effects with COS, as COS stimulated the *E. coli* growth. NDOs are capable of supporting bacterial growth, including xylo-oligosaccharides and pectic oligosaccharides, of specific gram-positive bacteria (32,33). In contrast, antibacterial properties of COS against several strains of gram negative and gram positive bacteria, such

as *S.* Typhimurium and *Bacillus. cereus* were observed [4,34]. These discrepancies might be related to the molecular weight, pH, bacterial strain and the degree of deacetylation and polymerization (34,35). Interestingly, we observed that low concentrations of AOS (0.5% and 1%) induce a remarkable *E. coli* growth inhibition (up to 30%). Khan et al (2012) pointed out that AOS (2%) can inhibit bacterial growth, including *P. aeruginosa* and *Acinetobacter. baumannii V19*, while an increase in *E. coli V5* 's density was observed by AOS (2%), significantly (17). This could be defined as strain-dependent and concentration-dependent effect of AOS. In our study, FOS did not exert a bacteriostatic effect on *E. coli*. Similarly, GOS up to 4% did not induce a change in *E. coli* growth, however, 6%, 8% and 10% GOS significantly inhibited *E. coli* growth. This might be attributed to the osmolarity changes induced by GOS, since *E. coli* can respond to changes in osmolarity of the growth medium (36). So far, no evidence regarding the inhibitory effects of GOS and FOS on *E. coli* growth has been reported. According to the above mentioned studies, it seemed that the different NDOs display a pathogen and concentration-dependent antimicrobial behavior. Different effects of these NDOs on *E. coli* growth could be related to their different chemical structures. GOS and FOS are neutral (37), while COS and AOS are positively and negatively charged, respectively (38–40). Considering these differences, it can be hypothesized that inhibition or stimulation of pathogen growth, could be linked to the multiple ionic interaction between charged oligosaccharides and the pathogenic exterior and flagella (41,42). For example, Parwell et al demonstrated that alginate oligomers, can induce a negative charge on gram-negative bacteria, via the direct binding to LPS and decrease bacterial motility and increase bacterial aggregation (43), highlighting the direct interaction of NDOs with the pathogen exterior (43,44). In addition to the effect of AOS on *E. coli* growth, AOS (0.5% and 1%) increased the sensitivity of *E. coli* to ampicillin as observed in an additional inhibitory effect on *E. coli* growth. A concentration of 0.5-1% AOS might reflect a realistic concentration to reduce the antibiotic concentration in order to inhibit the growth of a pathogen, such as *E. coli in vivo.* To our knowledge no clinical trials and *in vivo* studies have been conducted using AOS against *E.coli* infections, however, some *in vivo* studies investigated the effect of other NDOs on *E.coli* infection. In these studies, dosages in the range of 0.2-1% NDOs (FOS, mannan-oligosaccharides (MOS), and 2'-fucosyllactose (2'-FL)) were used in different species, including mice, chicken and pigs (45–50)(45–50)(45–50), which is in line with the AOS concentration used in our study. These studies showed that specific NDOs exhibit the capacity to attenuate the *E.coli*-induced adverse effects and *E.coli* growth *in vivo* (45–50). Thus, the challenge of the future will be to confirm the *in vivo* effectiveness of AOS and to identify the optimal dosing strategy.

He *et al* (2014) observed a synergistic effect of AOS in combination with a ribosome-targeting antibiotic on anti-biofilm capacity of gram-negative bacteria (*P. aeruginosa*) (15). However, the *E. coli* strain used in this study does not form biofilms. It is known that negatively charged AOS can strongly scavenge positive ions, such as Ca^{+2}. Calcium ions are involved in the preservation of bacterial cell structure, transport, motility and bacterial differentiation processes such as heterocyst formation, sporulation (51). It can be

suggested that AOS by chelating Ca^{+2} could affect the regular bacterial stability, which may increase the antimicrobial activity of ampicillin. Nevertheless, further research is needed to find the specific mechanism by which AOS inhibit *E. coli* growth and to investigate whether the observed effects in this study are strain/antibiotic-dependent. Within the last decades, numbers of molecular decoys were suggested to decrease the adherence of pathogens to intestinal epithelial cells, preventing the infection caused by these pathogenic bacteria (52,53). Several NDOs exhibited considerable anti-adhesive activity against intestinal pathogens (54,55). In this study, AOS also exerted an anti-adhesive effect on the *E. coli*. The mechanism of action for decreasing the adherence of *E. coli* to intestinal epithelial cells by AOS has not been clarified so far. It can be speculated that inhibited motility and the resulting bacterial aggregation as described above might have an inhibiting effect on the attachment of bacteria to epithelial cells (43). Interestingly, the combination of AOS with ampicillin further increased the anti-adhesive properties. This effect could be considered as the bactericidal effect of ampicillin in combination with the anti-adhesion capacity of AOS. In addition, this additional effect on anti-adhesion activity is most likely not related to the decrease in *E. coli* growth induced by AOS, since no significant changes in *E. coli* growth were observed after AOS treatment for 2 h (data not shown). COS, FOS and GOS did not display significant anti-adherence activity against the attachment of *E. coli* to HT-29 cells. In line with the present study, Shoaf *et al* (2006) (54) demonstrated that FOS did not exert a significant anti-adherence effect against *E. coli* in intestinal epithelial cells. Different studies indicated that the anti-adhesive properties of GOS and COS against *E. coli* are strongly strain-dependent (24,56). Several galactose units present in GOS can structurally mimic membrane glycans, which recognize and adhere to fimbrial and non-fimbrial adhesins expressed by intestinal pathogens (54,55,57,58). Investigations in recent years highlighted the anti-inflammatory effects of NDOs. In this study, pre-treatment with AOS, COS and GOS reduced the *E. coli*-induced pro-inflammatory cytokine (IL-8) response in HT-29 cells. The anti-inflammatory effect of these NDOs was also confirmed in other *in vitro* studies. LPS-induced inflammatory responses in microglial cells and macrophages can be remarkably reduced by AOS (59). Pretreatment with COS inhibited the LPS- and DSS-induced inflammatory responses in IPEC-J2 intestinal epithelial cells as measured by IL-6 and IL-8 levels (60) and COS downregulated the gene expression of different pro-inflammatory cytokines, including CCL15, CCL25 and IL1B, in Caco-2 cells (61). GOS suppressed the LPS-induced release of TNF-α, IFN-γ and IL-1β in human colon epithelial cells (62). In addition, GOS prevented the IL-8 expression and release in intestinal epithelium using an *in vitro* mycotoxin model (63). In the present study, FOS did not show a significant IL-8 reduction in HT-29 cells stimulated with *E. coli*. However, there are indications that immune modulation by different types of fructans is chain length- and source-dependent (64,65). However, the mechanisms by which NDOs exert immuno-modulatory effects have not been fully clarified. There are indications that several NDOs, such as GOS, COS and AOS are TLR4 ligands and most probably inhibit the phosphorylation of MAPKs and the activation of NF-κB in LPS-stimulated cells (27,66,67). Epithelial cells express proteins involved in the recognition of carbohydrate (glycan) structures, so called lectins, that might

also be involved in the anti-inflammatory properties of NDOs. One family of the soluble type lectins expressed by intestinal epithelial cells (IECs) are galectins, which exhibit binding specificity for β-galactosides (68,69). Intestinal epithelium-derived galectin-9 is involved in the immunomodulating effects of a GOS/FOS mixture (70). Zenhom M *et al* (2011) indicated that anti-inflammatory effect of oligosaccharides might be related to the induction of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), which regulates the peptidoglycan recognition protein 3 (PGlyRP3) (71).Moreover, the combinations of NDOs with ampicillin did not show any additional anti-inflammatory effects in this *in vitro* model, which could be related to the strong IL-8 inhibition by these NDOs, especially AOS and COS.

5. Conclusions

This *in vitro* study highlights the direct, microbiota-independent effects of NDOs on the *E. coli* growth, adhesion and *E. coli*-induced inflammatory response in intestinal epithelial cells. In particular, AOS, exhibiting anti-microbial, anti-adhesive and anti-inflammatory properties, might have the potential to be used in combination with ampicillin to decrease the ampicillin therapeutic concentration against *E. coli*. Further studies are warranted to investigate whether these observed effects induced by NDOs are strain and/or antibiotic dependent and to understand the mechanism of action by which NDOs can play a role in prevention of invasion and inflammation caused by *E. coli*.

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References

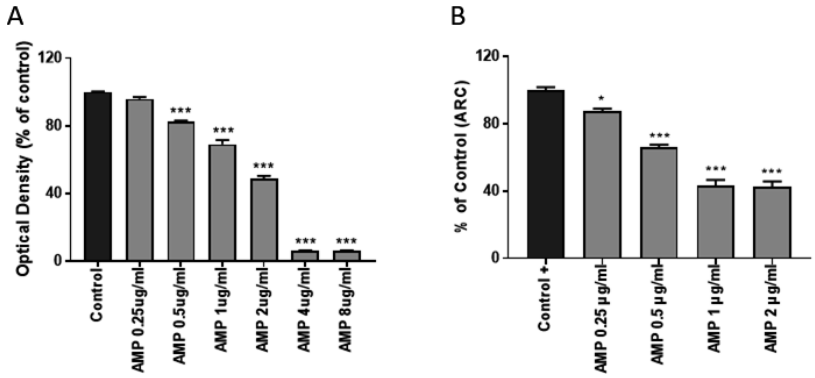
1. Sipahi OR. Economics of antibiotic resistance. Expert Rev Anti Infect Ther. 2008;6(4):523–39.
2. Venkatesan N, Perumal G, Doble M. Bacterial resistance in biofilm-associated bacteria. Future Microbiol. 2015;10(11):1743–50.
3. Summers AO. Genetic linkage and horizontal gene transfer, the roots of the antibiotic multi-resistance problem. Anim Biotechnol. 2007;5398:125–35.
4. Alanis AJ. Resistance to antibiotics: are we in the post-antibiotic era? Arch Med Res. 2005;36(6):697–705.
5. Walker AW, Lawley TD. Therapeutic modulation of intestinal dysbiosis. Pharmacol Res [Internet]. 2013;69(1):75–86. Available from: http://dx.doi.org/10.1016/j.phrs.2012.09.008
6. Ho J, Tambyah PA, Paterson DL. Multiresistant gram-negative infections: a global perspective. Curr Opin Infect Dis. 2010;23(6):546–53.
7. Andoh A. Physiological role of gut microbiota for maintaining human health. Digestion. 2016;93:176–81.
8. Holscher HD. Dietary fiber and prebiotics and the gastrointestinal microbiota. Gut Microbes [Internet]. 2017;8(2):172–84. Available from: http://dx.doi.org/10.1080/19490976.2017.1290756
9. Li P, Xia J, Nie Z, Shan Y. Pectic oligosaccharides hydrolyzed from orange peel by fungal multi-enzyme complexes and their prebiotic and antibacterial potentials. LWT - Food Sci Technol [Internet]. 2016;69:203–10. Available from: http://dx.doi.org/10.1016/j.lwt.2016.01.042
10. Gibson GR, Probert HM, Loo J Van, Rastall RA, Roberfroid MB. Dietary modulation of the human colonic microbiota : updating the concept of prebiotics. Nutr Res Rev. 2004;17(2):259–75.
11. Asadpoor M, Peeters C, Henricks PAJ, Varasteh S, Pieters RJ, Folkerts G, et al. Anti-pathogenic functions of non-digestible oligosaccharides in vitro. Nutrients. 2020 Jun;12(6):1789.
12. Li S, Li T, Zhu R, Wang N, Song Y, Wang S, et al. Antibacterial action of haw pectic oligosaccharides. Int J Food Prop. 2013 Jan;16(3):706–12.
13. Jeon YJ, Park PJ, Kim SK. Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr Polym. 2001 Jan;44(1):71–6.
14. Lin AE, Autran CA, Szyszka A, Escajadillo T, Huang M, Godula K, et al. Human milk oligosaccharides inhibit growth of group B *Streptococcus*. J Biol Chem [Internet]. 2017;292(27):11243–9. Available from: http://dx.doi.org/10.1074/jbc.M117.789974
15. He X, Hwang H, Aker WG, Wang P, Lin Y, Jiang X, et al. Synergistic combination of marine oligosaccharides and azithromycin against Pseudomonas aeruginosa. Microbiol Res [Internet]. 2014;169(9–10):759–67. Available from: http://dx.doi.org/10.1016/j.micres.2014.01.001
16. Powell LC, Sowedan A, Khan S, Wright CJ, Onsøyen E, Myrvold R, et al. The effect of alginate oligosaccharides on the mechanical properties of gram-negative biofilms. J Bioadhesion Biofilm Res. 2013;29(4):413–21.
17. Khan S, Tøndervik A, Sletta H, Klinkenberg G, Emanuel C, Onsøyen E, et al. Overcoming drug resistance with alginate oligosaccharides able to potentiate the action of selected antibiotics. Antimicrob Agents Chemother. 2012;56(10):5134–41.
18. Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R., & Swaminathan B. Salmonella nomenclature. J Clin Microbiol. 2000;38(7):2465–7.
19. Lane JA, Mehra RK, Carrington SD, Hickey RM. The food glycome : A source of protection against pathogen colonization in the gastrointestinal tract. Int J Food Microbiol [Internet]. 2010;142(1–2):1–13. Available from: http://dx.doi.org/10.1016/j.ijfoodmicro.2010.05.027
20. Rhoades J, Manderson K, Wells A, Hotchkiss A, Gibson G, Formentin K, et al. Oligosaccharide-mediated inhibition of the adhesion of pathogenic escherichia coli strains to human gut epithelial cells in vitro. J Food Prot. 2008;71(11):2272–7.
21. Yan YL, Hu Y, Simpson DJ, Ga MG. Enzymatic synthesis and purification of galactosylated chitosan oligosaccharides reducing adhesion of enterotoxigenic escherichia coli K88. J Agric Food Chem. 2017;65(25):5142–50.

22. Searle LEJ, Cooley WA, Jones G, Nunez A, Crudgington B, Weyer U, et al. Purified galactooligosaccharide , derived from a mixture produced by the enzymic activity of Bifidobacterium bifidum , reduces Salmonella enterica serovar Typhimurium adhesion and invasion in vitro and in vivo. J Med Microbiol. 2010;1428–39.
23. Quintero M, Maldonado M, Roberto MP, Hutkins R. Adherence inhibition of cronobacter sakazakii to intestinal epithelial cells by prebiotic oligosaccharides. Curr Microbiol. 2011;62(5):1448–54.
24. Rhoades J, Gibson G, Formentin K, Beer M, Rastall R. Inhibition of the adhesion of enteropathogenic Escherichia coli strains to HT-29 cells in culture by chito-oligosaccharides. Carbohydr Polym. 2006;64(1):57–9.
25. J. Garssen JFSVSB. Galacto-oligosaccharides exert a protective effect against heat stress in a Caco-2 cell model. J Funct Foods [Internet]. 2015;16:265–77. Available from: http://dx.doi.org/10.1016/j.jff.2015.04.045
26. Akbari P, Braber S, Alizadeh A, Verheijden KAT, Schoterman MHC, Kraneveld AD, et al. Galacto-oligosaccharides protect the intestinal barrier by maintaining the tight junction network and modulating the inflammatory responses after a challenge with the mycotoxin deoxynivalenol in human caco-2 cell. J Nutr. 2015;145(7):1604–13.
27. Ortega-gonz M, Ocon B, Romero-calvo I, Anzola A, Guadix E, Zarzuelo A, et al. Nondigestible oligosaccharides exert nonprebiotic effects on intestinal epithelial cells enhancing the immune response via activation of TLR4-NFκB. Mol Nutr Food Res. 2014;58(2):384–93.
28. Reed W, Williams R. Bacterial adherence: First step in pathogenesis of certain infections. J Chronic Dis. 1978;31:67–72.
29. Qu Y, Xu J, Zhou H, Dong R, Kang M, Zhao J. Chitin oligosaccharide (COS) reduces antibiotics dose and prevents antibiotics-caused side effects in Adolescent Idiopathic Scoliosis (AIS) patients with spinal fusion surgery. Mar Drugs. 2017;15(3):147–58.
30. Wang S, Wang J, Mou H. Inhibition of adhesion of intestinal pathogens (Escherichia coli, Vibrio cholerae, Campylobacter jejuni, and Salmonella Typhimurium) by common oligosaccharides. Foodborne Pathog Dis. 2015;12(4):360–5.
31. Ammar EM, Wang X, Rao C V. Regulation of metabolism in Escherichia coli during growth on mixtures of the non-glucose sugars : arabinose , lactose , and xylose. Sci Rep [Internet]. 2018;8(1):1–11. Available from: http://dx.doi.org/10.1038/s41598-017-18704-0
32. Li Z, Summanen PH, Komoriya T, Finegold SM, Li Z, Summanen PH, et al. In vitro study of the prebiotic xylooligosaccharide (XOS) on the growth of Bifidobacterium spp and Lactobacillus spp. Int J Food Sci Nutr. 2015;66(8):919–22.
33. Mandalari G, Palop CN, Tuohy K. In vitro evaluation of the prebiotic activity of a pectic oligosaccharide-rich extract enzymatically derived from bergamot peel. Appl Microbiol Biotechnol. 2007;73:1173–9.
34. Kyoon H, Young N, Ho S, Meyers SP. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. Int J Food Microbiol. 2002;74:65–72.
35. Kim S, Rajapakse N. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. Carbohydr Polym. 2005;62(4):357–68.
36. Record MT, Courtenay ES, Cayley DS, Guttman HJ. Responses of E . coli to osmotic stress : large changes in amounts of cytoplasmic solutes and water. Trends Biochem Sci. 1998;23(4):143–8.
37. Bakker-zierikzee AM, Alles MS, Knol J, Kok FJ, Tolboom JJM, Bindels JG. Effects of infant formula containing a mixture of galacto- and fructo- oligosaccharides or viable Bifidobacterium animalis on the intestinal microflora during the first 4 months of life. Br J Nutr. 2005;94(5):783–90.
38. Liaqat F, Eltem R. Chitooligosaccharides and their biological activities: A comprehensive review. Carbohydr Polym. 2018;184:243–59.
39. Powell LC, Pritchard MF, Ferguson EL, Powell KA, Patel SU, Rye PD, et al. Targeted disruption of the extracellular polymeric network of Pseudomonas aeruginosa biofilms by alginate oligosaccharides. npj Biofilms Microbiomes. 2018;4(1):1–10.

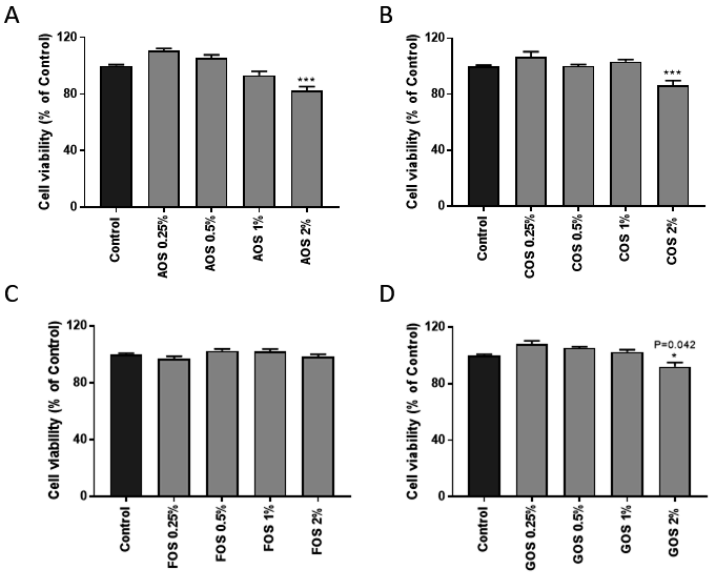
40. Pritchard MF, Powell LC, Jack AA, Powell K, Beck K, Florance H, et al. A low-molecular-weight alginate oligosaccharide disrupts pseudomonal microcolony formation and enhances antibiotic effectiveness. Antimicrob Agents Chemother [Internet]. 2017;61(9):1–14. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=A+Low-Molecular-Weight+Alginate+Oligosaccharide+Disrupts+Pseudomonal+Microcolony+Formation+and+Enhances+Antibiotic+Effectiveness
41. Chung Y, Su Y, Chen C, Jia G, Wang H, Wu JCG, et al. Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. Acta Pharmacol Sin. 2004;25(7):932–6.
42. Lambert P. Cellular impermeability and uptake of biocides and antibiotics in gram-positive bacteria and mycobacteria. J Appl Microbiol. 2002;92(S1):46S-54S.
43. Powell LC, Pritchard MF, Emanuel C, Onsøyen E, Rye PD, Wright CJ, et al. A nanoscale characterization of the interaction of a novel alginate oligomer with the cell surface and motility of pseudomonas aeruginosa. Am J Respir Cell Mol Biol. 2014;50:483–92.
44. Kumar ABV, Varadaraj MC, Gowda LR, Tharanathan RN. Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of papain and Pronase , and their bactericidal action against Bacillus cereus and Escherichia coli. Biochem J. 2005;175:167–75.
45. Yuanyifei Wang, Yan Zou, Jin Wang, Hui Ma, Bowei Zhang SW. The protective Effects of 2'-fucosyllactose against E. Coli O157 infection are mediated by the regulation of gut microbiota and the inhibition of pathogen adhesion. Nutrients. 2020;12:1284.
46. Baurhoo B, Phillip L, Ruiz-Feria CA. Effects of purified lignin and mannan oligosaccharides on intestinal integrity and microbial populations in the ceca and litter of broiler chickens. Poult Sci [Internet]. 2007;86(6):1070–8. Available from: http://dx.doi.org/10.1093/ps/86.6.1070
47. Wang W, Li Z, Han Q, Guo Y, Zhang B, D'Inca R. Dietary live yeast and mannan-oligosaccharide supplementation attenuate intestinal inflammation and barrier dysfunction induced by Escherichia coli in broilers. Br J Nutr. 2016;116(11):1878–88.
48. Baurhoo B, Ferket PR, Zhao X. Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. Poult Sci [Internet]. 2009;88(11):2262–72. Available from: http://dx.doi.org/10.3382/ps.2008-00562
49. Cilieborg MS, Sangild PT, Jensen ML, Østergaard M V., Christensen L, Rasmussen SO, et al. α1,2-Fucosyllactose does not improve intestinal function or prevent escherichia coli F18 diarrhea in newborn pigs. J Pediatr Gastroenterol Nutr. 2017;64(2):310–8.
50. Yang Y, Iji PA, Kocher A, Mikkelsen LL, Choct M. Effects of mannanoligosaccharide and fructooligosaccharide on the response of broilers to pathogenic Escherichia coli challenge. Br Poult Sci. 2008;49(5):550–9.
51. Jeon Y, Park P, Kim S. Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr Polym. 2001;44(1):71–6.
52. Ramirez-Hernandez A, Rupnow J, Hutkins RW. Adherence reduction of campylobacter jejuni and campylobacter coli strains to HEp-2 cells by mannan oligosaccharides and a high-molecular-weight component of cranberry extract. J Food Prot. 2015;78(8):1496–505.
53. Salminen A, Loimaranta V, Joosten JAF, Khan AS, Hacker J, Pieters RJ, et al. Inhibition of P-fimbriated Escherichia coli adhesion by multivalent galabiose derivatives studied by a live-bacteria application of surface plasmon resonance. J Antimicrob Chemother. 2007;60(3):495–501.
54. Shoaf K, Mulvey GL, Armstrong GD, Hutkins RW. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic escherichia coli to tissue culture cells. Infect Immun. 2006;74(12):6920–8.
55. Searle LEJ, Best A, Nunez A, Salguero FJ, Johnson L, Weyer U, et al. A mixture containing galactooligosaccharide , produced by the enzymic activity of Bifidobacterium bifidum , reduces Salmonella enterica serovar Typhimurium infection in mice. J Med Microbiol. 2009;37–48.
56. Sarabia-sainz HM, Armenta-ruiz C, Sarabia-sainz JA, Guzmán-partida AM, Ledesma-osuna AI, Vázquez-moreno L, et al. Adhesion of enterotoxigenic Escherichia coli strains to neoglycans synthesised with prebiotic galactooligosaccharides. Food Chem [Internet]. 2013;141(3):2727–34. Available from: http://dx.doi.org/10.1016/j.foodchem.2013.05.040

57. Sarabia-Sainz HM, Armenta-Ruiz C, Sarabia-Sainz JA, Guzman-Partida AM, Ledesma-Osuna AI, Vazquez-Moreno L, et al. Adhesion of enterotoxigenic Escherichia coli strains to neoglycans synthesised with prebiotic galactooligosaccharides. Food Chem. 2013;141(3):2727–34.
58. Quintero M, Maldonado M, Perez-Munoz M, Jimenez R, Fangman T, Rupnow J, et al. Adherence inhibition of cronobacter sakazakii to intestinal epithelial cells by prebiotic oligosaccharides. Curr Microbiol. 2011;62(5):1448–54.
59. Zhou R, Shi X, Bi D, Wei G, Xu X. Alginate-derived oligosaccharide inhibits neuroinflammation and promotes microglial phagocytosis of β-amyloid. Mar Drugs. 2015;13(9):5828–46.
60. Shi L, Fang B, Yong Y, Li X, Gong D, Li J, et al. Chitosan oligosaccharide-mediated attenuation of LPS-induced inflammation in IPEC-J2 cells is related to the TLR4/NF-κB signaling pathway. Carbohydr Polym [Internet]. 2019;219(April):269–79. Available from: https://doi.org/10.1016/j.carbpol.2019.05.036
61. Bahar B, Doherty JVO, Maher S, Mcmorrow J, Sweeney T. Chitooligosaccharide elicits acute inflammatory cytokine response through AP-1 pathway in human intestinal epithelial-like (Caco-2) cells. Mol Immunol [Internet]. 2012;51(3–4):283–91. Available from: http://dx.doi.org/10.1016/j.molimm.2012.03.027
62. Sun J, Liang W, Yang X, Li Q, Zhang G. Cytoprotective effects of galacto-oligosaccharides on colon epithelial cells. Life Sci [Internet]. 2019;231(59):116589. Available from: https://doi.org/10.1016/j.lfs.2019.116589
63. Akbari P, Braber S, Alizadeh A, Verheijden KAT, Schoterman MHC, Kraneveld AD, et al. Galacto-oligosaccharides protect the intestinal barrier by maintaining the tight junction network and modulating the inflammatory responses after a challenge with the mycotoxin deoxynivalenol in human caco-2 cell. J Nutr. 2015;145(7):1604–13.
64. Johnson-henry KC, Pinnell LJ, Waskow AM, Irrazabal T, Martin A, Hausner M, et al. Short-chain fructo-oligosaccharide and inulin modulate inflammatory responses and microbial communities in Caco2-bbe cells and in a mouse model of intestinal injury. J Nutr. 2014;144(11):1725–33.
65. Vogt L, Ramasamy U, Meyer D, Pullens G, Venema K, Faas MM, et al. Immune modulation by different types of b 2 R 1-fructans is toll-like receptor dependent. PLoS One. 2013;8(7):1–12.
66. Zhou R, Shi X, Gao Y, Cai N, Jiang Z, Xu X. Anti-inflammatory activity of guluronate oligosaccharides obtained by oxidative degradation from alginate in lipopolysaccharide-activated murine macrophage RAW 264.7 cells. J Agric Food Chem. 2015;63(1):160–8.
67. Li Y, Liu H, Xu Q, Du Y, Xu J. Chitosan oligosaccharides block LPS-induced O-GlcNAcylation of NF-κB and endothelial inflammatory response. Carbohydr Polym. 2014;99:568–78.
68. Kivit S De, Kraneveld AD, Garssen J, Willemsen LEM. Glycan recognition at the interface of the intestinal immune system : Target for immune modulation via dietary components. Eur J Pharmacol [Internet]. 2011;668:S124–32. Available from: http://dx.doi.org/10.1016/j.ejphar.2011.05.086
69. Pieters RJ. Inhibition and detection of galectins. ChemBioChem. 2006;7(5):721–8.
70. Kivit S De, Kraneveld D, Knippels LMJ. Intestinal epithelium-derived galectin-9 is involved in the immunomodulating effects of nondigestible oligosaccharides. J Innate Immun. 2013;5:625–38.
71. Zenhom M, Hyder A, Vrese M De, Heller KJ, Roeder T. Prebiotic oligosaccharides reduce proinflammatory cytokines in intestinalcCaco-2 cells via activation of PPARγ and peptidoglycan recognition protein 3. J Nutr. 2011;141(5):971–7.

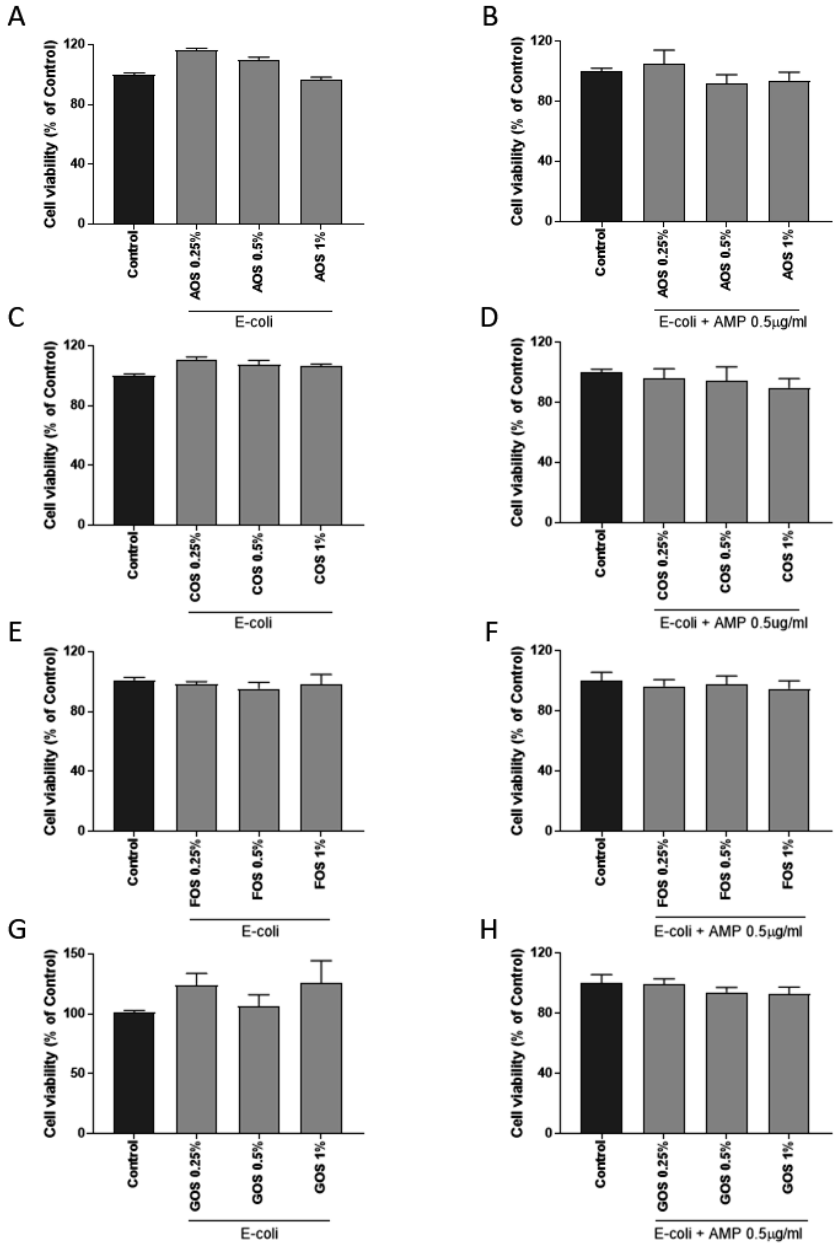
Supplementary Figures



Supplementary figure 1. The effects of ampicillin on E. coli growth and adherence of E. coli to HT-29 cells. E. coli was grown overnight in presence or absence of ampicillin and OD measurements (MIC assay) were used for determination of E. coli growth (a). HT-29 cells exposed to E. coli in presence or absence of ampicillin for 2 h, were lysed and seeded on blood agar and colonies were counted (b). Results are expressed as relative bacterial growth (a) or adhesive rate constant (ARC) (percentage of bacteria adhered relative to control) (b) as mean ± SEM of three independent experiments each performed in triplicate. * = P<0.05 compared to control. *** = P<0.001 compared to control).



Supplementary figure 2. The viability of HT-29 cells exposed to NDOs for 24h. HT-29 cells were pre-treated with different concentrations (0.25%, 0.5%, 1% and 2%) AOS (a), COS (b), FOS (c) and GOS (d) for 24 h and cell viability was determined via the MTT assay. The results are expressed relative to the control cell number as mean ± SEM of three independent experiments each performed in triplicate. (* = P<0.05 compared to control. *** = P<0.001 compared to Control). Abbreviations: AMP, ampicillin; AOS, alginate oligosaccharides; COS, chitosan oligosaccharide; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.



Supplementary figure 3. The viability of HT-29 cells exposed to NDOs and challenged with E. coli in presence or absence of ampicillin. HT-29 cells were pre-treated with different concentrations (0.25%, 0.5%, 1% and 2%) AOS (a, b), COS (c, d), FOS (e, f) and GOS (g, h) for 24 h followed by a challenge with E. coli in presence (b, d, f, h) or absence (a, c, e, g) of ampicillin (0.5 μg/mL) for 4h and cell viability was determined via the MTT assay. The results are expressed relative to the control cell number as mean ± SEM of three independent experiments each performed in triplicate. Abbreviations: AMP, ampicillin; AOS, alginate oligosaccharides; COS, chitosan oligosaccharide; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.

Chapter 4

Antimicrobial activities of alginate and chitosan oligosaccharides against *Staphylococcus aureus* and Group B *Streptococcus*

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Abstract

The bacterial pathogens *Streptococcus agalactiae* (GBS) and *Staphylococcus aureus* (*S. aureus*) cause serious infections in humans and animals. The emergence of antibiotic-resistant isolates and bacterial biofilm formation entails the urge of novel treatment strategies. Recently, there is a profound scientific interest in the capabilities of non-digestible oligosaccharides as antimicrobial and anti-biofilm agents as well as adjuvants in antibiotic combination therapies. In this study, we investigated the potential of alginate oligosaccharides (AOS) and chitosan oligosaccharides (COS) as alternative for, or in combination with antibiotic treatment. AOS (2 - 16 %) significantly decreased GBS V growth by determining the minimum inhibitory concentration. Both AOS (8 and 16 %) and COS (2 - 16 %) were able to prevent biofilm formation by *S. aureus* wood 46. A checkerboard biofilm formation assay demonstrated a synergistic effect of COS and clindamycin on the *S. aureus* biofilm formation, while AOS (2 and 4%) were found to sensitize GBS V to trimethoprim. In conclusion, AOS and COS affect the growth of GBS V and *S. aureus* wood 46 and can function as anti-biofilm agents. The promising effects of AOS and COS in combination with different antibiotics may offer new opportunities to combat antimicrobial resistance.

Keywords*: Alginate oligosaccharides, Chitosan oligosaccharides, S. Aureus, Group B Streptococcus, Bacterial growth, Anti-biofilm, Synergy, Sensitization.*

1 Introduction

Among various pathogenic agents, *Staphylococcus aureus* (*S. aureus*) and Group B *Streptococcus* (GBS), alternatively called *Streptococcus agalactiae* (*S. agalactiae*), cause serious infections in both humans and animals at a global scale. These pathogens can cause a wide spectrum of invasive diseases ranging from neonatal sepsis, meningitis and pneumonia to severe mastitis in cattle (1,2). Both pathogens produce multiple virulence factors and have the capability to form biofilms (3,4). An increasing problem in treating these infections is the emergence of strains that are resistant to antimicrobial treatment (5,6). Infections related to the pathogenic form of GBS might occur in utero or with its passage through the birth canal during parturition. The percentage of neonates from GBS-colonized mothers that become transiently colonized with GBS by their mother's organism is about 30 to 70 % (7). Despite its role as a common intestinal colonizer in infants, how GBS retains its potential virulence and its transition from a commensal to a devastating pathogen remains poorly understood (8). On the other hand, *S. aureus* causes a wide range of diseases, such as toxic shock syndrome, infective endocarditis, as it is capable to disrupt tissue barriers, entering the bloodstream, and contaminating almost every organ in the body (9). Additionally, *S. aureus* is the most common causative pathogen of infectious mastitis that might appear at every stage of life, but occurs mostly in women during the breast-feeding period. The global incidence of mastitis within lactating women varies from 1 to 10 %, although some studies indicate that this infection can be observed to reach 33 % of lactating women (10). Through breastfeeding, *S. aureus* can be transferred to the gut microflora of newborns where it colonizes the gastrointestinal tract (11).

The high prevalence of pediatric infectious diseases linked with the colonization and the pathogenicity of GBS and *S. aureus* in the gastrointestinal tract of infants, has increased the attention towards alternative approaches to prevent/reduce the incidence of these infections. Misuse and overuse of broad-spectrum antibiotics has resulted in a situation wherein bacteria promote the evolution of phenotypes resistant to nearly every antibiotic in clinical use (12). Therefore, there is an urgent need for alternatives that can tackle the problem of antimicrobial resistance. Antibiotic combination therapy, which involves the co-administration of antibiotics with an adjuvant that suppresses the resistance and enhances the antibiotic function and efficacy, offers promising therapeutic perspectives (13). One of the mechanisms that bacteria use to develop resistance to antibiotics is based on the alteration of their physiology through the formation of a biofilm matrix. It is estimated that 65 % of all bacterial infections result in bacterial biofilm formation (6), one of the interesting characteristics of many bacteria, including *S. aureus* and GBS (3,4). Especially, biofilm formation on implanted materials and medical devices, such as catheters, endotracheal tubes, and prosthetic joints, poses a serious public health problem (6).

The composition and the stability of biofilms is dependent on the structure of their extracellular polymeric substances (EPS), a matrix that is mainly composed of

polysaccharides, proteins, lipids, and extracellular DNA (eDNA) (14). Different hypotheses have been examined to explain the antimicrobial persistence in the unbreakable structures of biofilms. First, it is believed that antibiotics can be inactivated by antibiotic-degrading enzymes, which are accumulated in the biofilm matrix. Second, the low metabolic activity of microorganisms observed in a biofilm is correlated with antibiotic tolerance (15). Additionally, the intrinsic structure of biofilms may prevent the antibiotics to penetrate into the biofilm due to a high abundance of water channels (15,16). Given the complex mechanisms responsible for antibiotic resistance of bacteria in biofilms, a combination of various defensive ways may be needed to successfully combat these bacterial structures.

Non-digestible oligosaccharides (NDOs), complex carbohydrates that resist hydrolysis by salivary and intestinal digestive enzymes and known for their prebiotic properties by stimulating beneficial bacteria in the gut microbiota, These NDOs also exhibit various pathogen reduction capabilities, as reviewed in Asadpoor et al. (17,18), and thus may represent potential therapeutic candidates against infections. NDOs, such as human milk oligosaccharides (HMOs), chitosan oligosaccharides (COS) and alginate oligosaccharides (AOS), two NDOs that structurally resemble HMOs, can exhibit anti-biofilm activity (17,19,20), not only by preventing biofilm formation, but also by decomposing preformed biofilms probably via the disruption of EPS components (19). Furthermore, specific NDOs, and especially HMOs, exert a bacteriostatic effect on bacterial growth (18,21). Based on different investigations, AOS and COS have already shown anti-virulence and anti-biofilm properties against different bacteria, such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (22,23).

Given the antimicrobial capacity of AOS and COS, the current study investigated the potential of these two promising NDOs to inhibit bacterial growth and biofilm formation of *S. aureus* and GBS. In addition, the effect of the NDOs in combination with antibiotics was tested to evaluate possible synergistic effects that may diminish the bacterial resistance to antimicrobials.

2 Materials and methods

2.1 Bacterial strains and culture conditions

S. aureus strain wood 46 (ATCC 10832) (24) and wild-type (WT) GBS clinical isolate NCTC 10/84 (1169-NT1; ATCC 49447) (serotype V) (25) were gifts from Suzan Rooijakkers (UMC, University Medical Center, Utrecht, The Netherlands) and Nina van Sorge (AMC, Academic Medical Center, Amsterdam, The Netherlands), respectively. Bacteria were stored at - 80°C. For all experiments, bacteria were first grown on blood agar plates (Biotrading, Mijdrecht, The Netherlands) at 37°C for 24 h and subsequently, the colonies were sub-cultured in tryptic soy broth (TSB) and incubated overnight at 37°C under shaking conditions (160 rpm). After incubation, bacterial growth (OD_{600}) was measured and bacterial density

was adjusted according to OD600 = 0.5 (determination of the minimum inhibitory concentration) or at OD600 = 1 (biofilm formation assay).

2.2 AOS and COS (NDOs)

The NDOs, AOS (purity > 85 %) and COS (purity > 90 %), were purchased from BZ Oligo Biotech Co., Ltd. (Qingdao, Shandong, China). AOS was produced by the degradation of algin and COS originated from marine biological sources (shrimp and crab shells). Their structures are depicted in **Figure 1A**. NDO (AOS and COS) solutions were freshly dissolved in TSB before each experiment and the pH of the solution was adjusted to 7.2 – 7.4. The chemical NDO structures were drawn using ChemDraw Professional 15.0.

2.3 Antibiotics

Ampicillin (AMP), Ciprofloxacin (CIP), Clindamycin (CLI), Tetracycline (TET) and Trimethoprim (TMP) were purchased from Sigma-Aldrich (Steinheim, Germany). These five common-used antibiotics were chosen based on a variety of chemical structures (**Figure 1B**) and mechanisms of action, to assess whether in combination with AOS and COS can sensitize bacterial strains (*S. aureus* strain wood 46 and GBS V) to these antibiotics. Sterile stock concentrations of each antibiotic were made in TSB and used for serial dilutions and prior to each experiment fresh stocks were prepared. The chemical antibiotic structures were drawn using ChemDraw Professional 15.0.

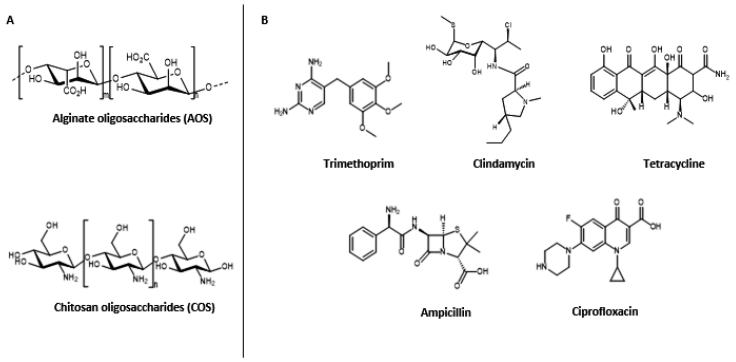

Figure 1. Structures of oligosaccharides and antibiotics used for antimicrobial activity against GBS V and *S. aureus* wood 46. (A) Structures of AOS and COS. (B) Structures of bacteriostatic (TMP, CLI, TET) and bactericidal (AMP, CIP) antibiotics.

2.4 Determination of the minimum inhibitory concentration

The antibacterial capacity of AOS and COS against GBS V and *S. aureus* wood 46 was determined via analyzing the minimum inhibitory concentration following the method as described previously (26). AOS and COS were serially diluted in 96-well U-bottom polypropylene plates (Corning Costar, Cambridge, MA, USA) to reach 100 µL final volume with concentrations ranging from 16 % to 0.25 %. Subsequently, 100 µL of bacterial inoculums (*S. aureus* strain wood 46 and GBS V) with OD600 = 0.5 (approximately10^{+8}colony

forming units [CFU]/ml) were added to serially diluted NDOs (27,28). The plates were covered with sterile breathable film (VWR International, Amsterdam, Netherlands) and incubated overnight at 37 °C under shaking conditions (160 rpm). After the incubation period, 100 µL of culture medium was transferred to 96-well F-bottom polystyrene microtiter plates (Corning Costar, Cambridge, MA, USA) and the signal was measured at 600 nm with a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany). Bacteria growth in TSB without treatment served as positive control and TSB alone was used as negative control. The minimum inhibitory concentration was considered as the lowest concentration that inhibits bacterial growth by more than 90 % in comparison to positive control groups, as IC_{90} value correlated well with the minimum inhibitory concentration of a compound as described by (29,30).

2.5 Antibiotic sensitization assay

The ability of AOS to sensitize the GBS strain V to specific antibiotics was determined using the antibiotic sensitization assay as described before (31). AOS were serially diluted in 96-well U-bottom polypropylene plates with concentrations ranging from 16 % to 2 %. Subsequently, AOS was combined with different concentrations of AMP, CLI, TET and TMP. The concentration range of the studied antibiotics was chosen based on the determination of the minimum inhibitory concentration. Concentrations below the minimum effective concentration were selected in order to create a wide range, in which the additional effect of AOS could be visible. Thereafter, 100 µL bacterial inoculums with OD = 0.5 was added to the treatments. Plates were covered with sterile breathable film (VWR International, Amsterdam, Netherlands) and incubated for 24 h at 37 °C under shaking conditions (160 rpm). After the incubation period, the optical density was measured with a FLUOstar Omega microplate at 600 nm. Bacteria growth in TSB without any treatment served as positive control, while wells with TSB (no treatment) were considered as negative control. For determining statistical significance, the outcome of the combinational treatment was compared to both the results of corresponding antibiotic concentration and corresponding AOS concentration. Therefore, the final effect was considered statistically significant when all two conditions were significant different compared to the combination therapy, suggesting sensitization was achieved.

2.6 Biofilm formation assay

The biofilm formation assay was performed for the evaluation of the effect of AOS and COS on the biofilms produced by *S. aureus* wood 46 and GBS V. As described above, for the biofilm formation assay, bacteria strains were grown in TSB at 37 °C under shaking conditions (160 rpm). Afterwards, different procedures were carried out for the growth of streptococci and staphylococci biofilms.

For the development of streptococci biofilm, a biofilm formation assay was adapted from methods described previously (32). Briefly, NDO treatments of serially diluted concentrations (16 - 0.5 %) were prepared in 96-well F-bottom polystyrene microtiter

plates. For the preparation of the serial dilutions of AOS and COS, the biofilm medium (BM) composed of TSB supplemented with 0.5 % glucose and 3 % NaCl was used. Thereafter, the optical density of GBS V was adjusted at OD600 = 0.5 and 100 µL of the inoculated medium was transferred into the 96-well plates in the presence of increasing NDO concentrations. The biofilms were grown for 24 h at 37 °C with 5 % CO_2 under static conditions. Furthermore, bacteria grown in BM in the absence of any intervention served as positive control, representing the maximum biofilm growth. Uninoculated culture media (BM: TSB, 1:1) was considered as negative control.

For the biofilm formation by staphylococci, a different procedure was followed as described by Kang et al. (33). Briefly, the OD of grown bacteria was adjusted at OD600 = 1. To prepare the working bacterial solution (WBS), 10 µL of bacterial solution was added to 10 mL of BM (1:1000). NDO treatments were prepared in 96-well F-bottom plates of serial diluted concentrations (16 - 0.5 %). Thereafter, 100 µL WBS was added in 96-well F-bottom plates in absence or presence of NDOs and incubated for 24 h at 37 °C with 5 % CO_2 under static conditions. For quantifying the biofilm inhibitory effect of NDOs, full-formed biofilms without any additional treatment were used as positive control and uninoculated culture media (BM: TSB, 1:1) was used as negative control.

Subsequently, supernatants of both streptococci and staphylococci biofilms were gently removed, wells were washed with 200 µL of phosphate-buffered saline (PBS) and the bacterial biofilms were fixed at 60 °C for 30 min. The fixed biofilms were stained with 160 µL of crystal violet (CV) solution (0.1 %) for 5 min. Excess stain was discarded and wells were washed twice with tap water. Stained biofilms were solubilized in 160 µL of acetic acid (33 %) and 100 µL was gently transferred to a 96-well F-bottom plate. The biofilm formation was measured at 595 nm using a FLUOstar Omega microplate reader.

Minimum biofilm inhibitory concentration (MBIC) of NDOs against GBS V and *S. aureus* wood 46 was determined as the lowest concentration of NDOs that inhibit biofilm formation by more than 90 % in comparison to control groups (29,30).

2.7 Checkerboard biofilm formation assay

The checkerboard biofilm formation assay was performed in order to identify the type of interaction (synergistic, additive, indifferent, or antagonistic) between AOS and COS with the five antibiotics against bacterial biofilm formation by S. aureus (34). Concentration ranges of antibiotics are depicted in Table 1.

Serial dilutions of NDOs were prepared with concentrations ranging from 16 % to 0.25 % and were added to the different rows of the flatbottom 96 well plate (Figure 5A). The antibiotic dilutions were added to the different columns of the 96 well plate (Figure 5A). In this regard, a variety of mixtures of different concentrations of the assessed compounds (NDOs and antibiotics) was created with controls for NDOs (far-right column) and

antibiotics (bottom row). The well at the bottom right corner was used as positive control, in which only inoculated medium was present in order to indicate the maximum biofilm growth. Subsequently, biofilms were prepared and biofilm formation was measured as described in section 2.5. Briefly, 100 µL of the WBS was added to the combined treatments to reach 200 µL final volume per well. The plate was incubated at 37°C with 5 % CO_2 for 24 h at static conditions. Following the incubation period, the medium was gently aspirated and the wells were washed once with 200 µL PBS to remove free-floating "planktonic" bacteria. After the washing procedure, the biofilm was fixed at 60 °C for 30 min. For measuring the biofilm biomass, the fixed biofilm was stained with 160 µL of CV solution (0.1 %) for 5 min and the excess stain was eliminated by two washes with tap water. For solubilizing the bound CV, 160 µL of acetic acid (33 %) was added to the wells and the optical absorbance was determined at 595 nm using a FLUOstar Omega microplate reader.

In this study, the nature of the interaction of the combinational agents was evaluated by determination of the anti-biofilm capacity of the agents and using the fractional biofilm inhibitory concentration (FBIC). For calculating FBIC index, the observing equation was used: ΣFBIC = FBIC A + FBIC B = (MBIC of drug A in the combination / MBIC of drug A alone) + (MBIC of drug B in the combination / MBIC of drug B alone) (35,36). The lowest ΣFBIC index was chosen for the strongest interaction between two agents. The effect of two agents is considered as synergy when the ΣFBIC index is 0.5 or less, additive when the ΣFBIC index is between 0.5 and 1, indifferent when the ΣFBIC index is between 1 and 4, and antagonistic when the ΣFBIC index is 4 or more (36,37). Synergy means the interaction or cooperation of two or more organizations, substances, or other agents to produce a combined effect greater than the sum of their separate effects. Additive means the overall consequence, which is the result of two agents acting together and which is the simple sum of the effects of the agents acting independently. Indifferent means the combination has no increase in inhibitory activity of both agents. Antagonistic means the effect produced by the contrasting actions of two (or more) agents (38).

To assess the effectivity of the most optimal combination (COS and CLI) on bacterial growth inhibition, the supernatant of the combination of treatments that acquired the best inhibitory effect and the lowest ΣFBIC was collected and grown on blood agar plates. One loopful (approx. 10 µL) of the corresponding well was transferred into a separate blood agar plate. The transferred amount was evenly spread over the surface and the agar plate was incubated at 37 °C for 24 h. The bacterial growth of the combinational treatment was optically compared with the growth of bacteria with each agent separately as well as with the positive control (without treatment).

Table 1. Minimum biofilm inhibitory concentration (MBIC) of *S. aureus* wood 46 and ranges of antibiotic solutions performed in the checkerboard biofilm formation assay.

Antibiotics	MBIC (µg/mL)	Ranges of tested concentrations (µg/mL)
AMP	0.0156	0.0128 - 0.0002
CIP	0.5	4 – 0.0078
CLI	0.25	2 – 0.0039
TET	0.25	2 – 0.0039
TMP	2	8 – 0.0156

2.8 Statistical Analysis

Data were reported as mean values ± SEM of at least three independent experiments (n=3) routinely performed in triplicate (3 wells / condition). Results were analyzed using Prism 8.0 GraphPad Software (GraphPad, San Diego, CA, USA). Statistical significance was determined using ANOVA followed by Bonferroni post-hoc test. Differences were considered statistically significant when $P < 0.05$.

3 Results

3.1 AOS and COS differentially affect *S. aureus* and GBS V growth

To evaluate whether AOS and COS can inhibit the growth of the two pathogenic strains, GBS V and *S. aureus* wood 46, bacterial growth in TSB in the absence and presence of increasing concentrations of AOS and COS was investigated. As shown in Fig. 2, the addition of AOS caused a significant concentration-dependent reduction in the growth of GBS V. At AOS concentrations below 2 % (0.25, 0.5 and 1 %) no significant inhibition of GBS V growth was observed (**Figure 2A**). Although higher AOS concentrations decreased the growth of GBS V, the minimum inhibitory concentration was not identified, since the growth inhibition did not reach 90% or higher. Maximum inhibition of growth (81%) was achieved at concentration of 8% AOS. Unlike AOS, COS treatment (0.25-8%) did not inhibit, but even enhanced GBS V growth by up to 2-fold. Using the highest COS concentration (16%), a slight but not significant reduction in GBS V growth was measured (**Figure 2B**). Similar measurements of the effects of AOS and COS on the growth of *S. aureus* demonstrated unaltered growth of *S. aureus* in the presence of AOS (**Figure 2C**), whereas COS (0.25-8%) again caused an increase in bacterial (*S. aureus*) growth (**Figure 2D**).

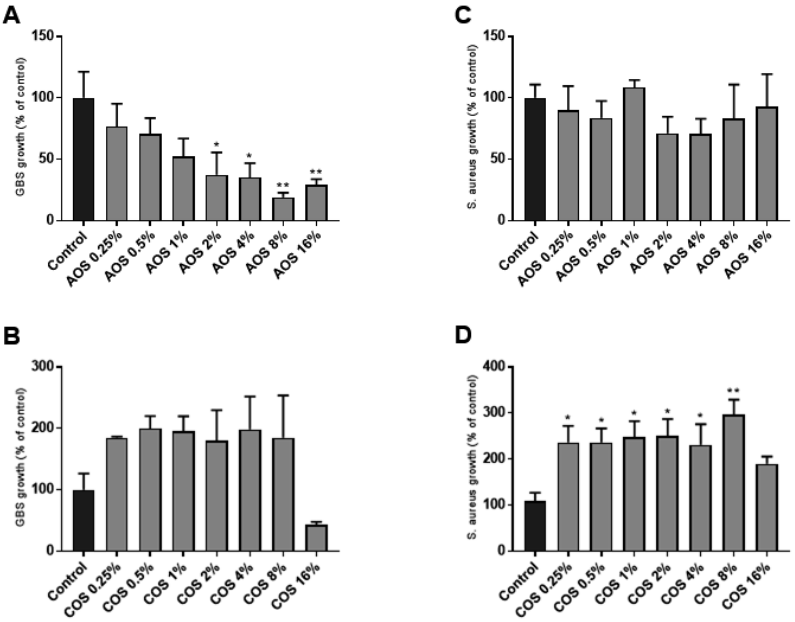

Figure 2. Effect of AOS and COS on bacterial growth of GBS V and *S. aureus* wood 46 strains in TSB. In order to identify the MIC of AOS and COS against GBS V (**Figure 2A, 2B**) and *S. aureus* wood 46 (**Figure 2C, 2D**), seven 2-fold serial dilutions of each NDO were examined, as described in the material and methods section. Control represents the percentage of the maximum growth of bacteria without any intervention. Results are expressed as the percentage of bacterial growth (relative to control) as mean ± SEM of three independent experiments each performed in triplicate. Statistical differences *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$) compared to positive control were obtained using one-way ANOVA test. AOS, alginate oligosaccharides; COS, chitosan oligosaccharides.

3.2 AOS differentially affect the sensitization of GBS V towards 4 different antibiotics (AMP, CLI, TET, TMP)

In order to investigate whether AOS is able to sensitize GBS V to antibiotics (AMP, CLI, TET and TMP), an antibiotic sensitization assay was conducted. Hereto, GBS V growth in the absence and presence of increasing concentrations of AOS and antibiotics was followed over time. A downward trend in GBS V growth is observed by all the AOS-antibiotic combinations tested, however, AOS-AMP and AOS-TET did not provoke any GBS sensitization to the antimicrobial agents, since any of combinational concentrations did not significantly decrease the bacterial growth comparing with corresponding concentration of AOS as well as with the antibiotic (**Figure 3A, 3C**). Interestingly, a striking reduction in the minimum inhibitory concentration of CLI was visible when AOS was combined. When 4% AOS was added to 0.0313 ug/mL CLI, the same reduction of GBS growth was observed as obtained with a 3 times higher CLI concentration (0.125 ug/mL) without AOS supplementation **(Figure 3B)**. Furthermore, AOS sensitized GBS V to the highest resistance concentrations of TMP (8 - 64 µg/mL) (**Figure 3D)**. More specifically, 2 and 4 % AOS induced a significant

reduction of TMP at concentrations more than 8 µg/mL, and from 8 µg/mL to 32 µg/mL, respectively **(Supplementary figure 1**).

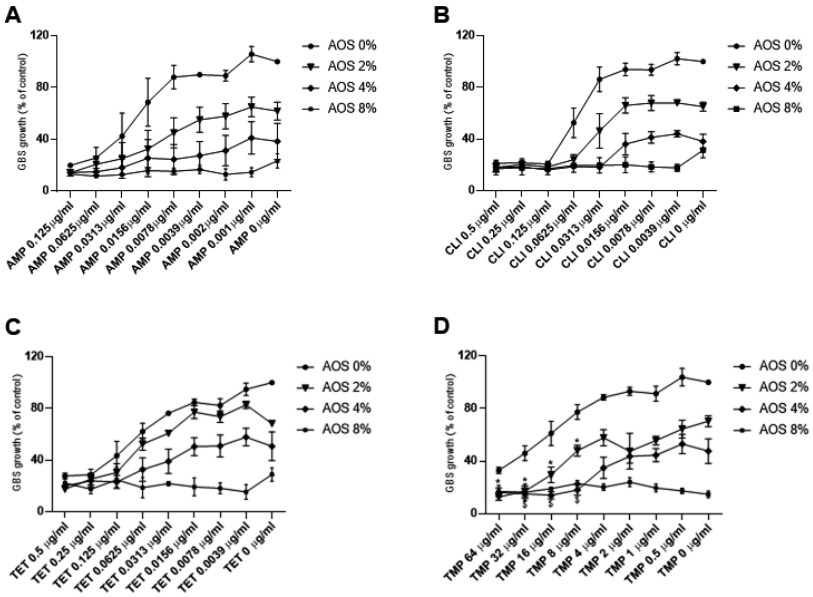

Figure 3. Effect of AOS in combination with AMP, CLI, TET and TMP on the growth of GBS V strain. To examine whether AOS has the feasibility to sensitize GBS V to antibiotics, a sensitization assay was performed as described in the material and method section. AOS (2, 4 and 8 %) were combined with different concentrations of AMP (**Figure 3A**), CLI (**Figure 3B**), TET (**Figure 3C**) and TMP (**Figure 3D**). Sensitization was only achieved when 2 % and 4% AOS were combined with TMP. Star (for 2% AOS) or dollar (for 4% AOS) are representing a significant reduction of the combinational treatments comparing with both corresponding antimicrobial agents (AOS and antibiotics). Positive control represents the percentage of the absolute growth of bacteria (100 % growth) without the presence of any treatment. The results are expressed as the percentage of bacterial growth as mean ± SEM of three independent experiments each performed in a minimum of 3 replicates. Statistical differences *, $ ($P < 0.05$) compared to control and the corresponding concentrations of antibiotics and AOS, were obtained using a two-way ANOVA test.

3.3 AOS and COS induce different changes in biofilm formation by GBS and *S. aureus*.

To assess the behavior of AOS and COS during biofilm formation, the biofilm-forming capacity of GBS V and *S. aureus* was conducted in presence and absence of AOS or COS by staining with CV. As shown in **Figure 4A**, the highest concentration of AOS (16 %) induced a significant reduction (71%) of biofilm formation by GBS V compared to the positive control (without treatment). This cannot been considered as MBIC, since this inhibition did not reach 90% or higher. COS treatment did not affect the GBS V biofilm formation (**Figure 4B)**. In contrast, both AOS and COS showed an inhibitory effect on *S. aureus* biofilm formation. AOS showed an inhibitory effect in a concentration-dependent manner, although only at 8 % AOS and 16 % AOS the effects were statistically significant, as

depicted in **Figure 4C.** With the highest AOS concentration (16 %) even a 90% inhibition of biofilm formation was achieved (MBIC). COS (2-16 %) treatment significantly reduced the biofilm formation of *S. aureus* wood 46. Precisely, COS 16 % and COS 8 % (MBIC) exhibited 97 % and 90 % inhibition of *S. aureus* biofilm formation, respectively. At concentrations below 2% COS no effect was observed (**Figure 4D**).

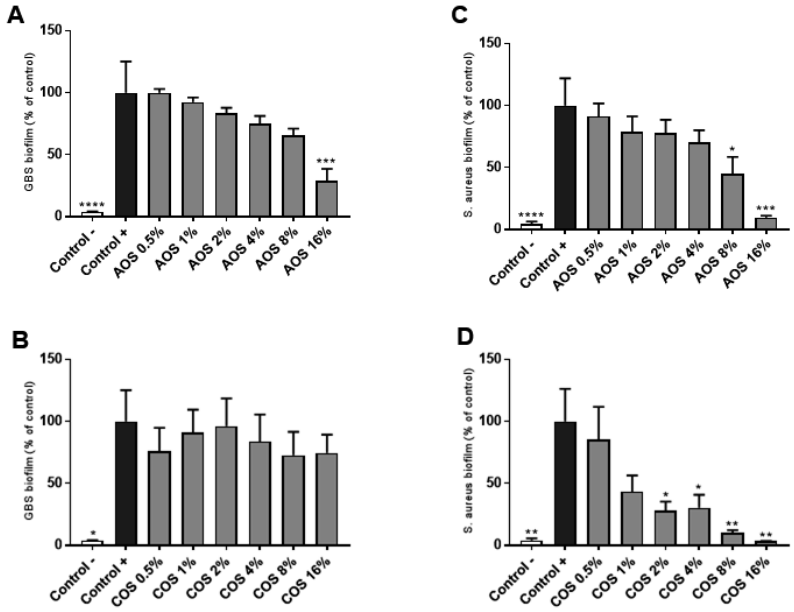

Figure 4. Anti-biofilm activity of AOS and COS against GBS V and *S. aureus* wood 46 strains. For the biofilm formation assay, six 2-fold serial dilutions of AOS and COS were tested after 24h of exposure, targeting the MBIC against GBS V (**Figure 4A, 4B**) and *S. aureus* wood 46 (**Figure 4C, 4D**), as described in the material and methods section. Control (-) represents the negative control (uninoculated culture media without NDO treatment) and control (+) represents full-formed biofilms without any additional treatment 16 % AOS and 8 % COS are considered as MBIC against *S. aureus* wood 46. Results are expressed as the percentage of biofilm formation (relative to positive control) as mean ± SEM of three independent experiments each performed in triplicate. Statistical differences *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) and ****($P < 0.0001$) compared to positive control were obtained using one-way ANOVA test. AOS, alginate oligosaccharides; COS, chitosan oligosaccharides.

3.4 Differential interactions of COS and AOS with 5 different antibiotics (AMP, CLI, CIP, TET, TMP) against biofilm formation of *S. aureus*

To test the possible additive effects of NDOs on the action of conventional antibiotics, a biofilm formation checkerboard assay was performed (Table 2). The different interactions were measured using the FBIC index as described in the material and methods section. Among different combinations tested, COS with CLI obtained a synergistic anti-biofilm effect. As shown in **Figure 5A**, a simulation of the checkerboard biofilm assay is depicted, demonstrating the synergistic effect of 2 % COS in combination with 0.0625 µg/mL CLI (FBIC value, 0.5) on *S. aureus* biofilm formation. This specific combination resulted in the

lowest FBIC index among the different combinations that exhibited full inhibition of *S. aureus* biofilm formation. Along with the FBIC value related to synergy, the FBIC values of the wells, in which an additive effect was reported, were also calculated and mentioned in **Figure 5A**. Concerning the other antibiotics, an additive effect was reported with the treatments of AOS and COS in conjunction with TET, and the FBIC values were 0.54 and 0.75, respectively. Additive interaction was also observed when AOS was combined with CLI (FBIC value, 0.73). The nature of interaction of both AOS and COS with CIP (FBIC value, 1.01 and 1.03, respectively) or TMP (FBIC value, 1.01 for both) was characterized as indifferent. Finally, antagonism interaction was reported when AMP was combined with COS (FBIC value > 4) and indifference when combined with AOS (FBIC value, 1.42).

Furthermore, the supernatant of the combinational treatment that achieved the most optimal effect (COS with CLI, FBIC value, 0.5) was collected and cultured on blood agar plates in order to identify whether the two agents could also reduce the bacterial growth in addition to the full inhibition of *S. aureus* biofilm formation. As depicted in **Figure 5F**, the *S. aureus* colonies treated with the COS-CLI combination were almost eliminated in comparison with the number of *S. aureus* bacteria in positive control group (**Figure 5B**), treated with 0.0625 μg/mL CLI (**Figure 5C**) or 2 % COS (**Figure 5D**).

Table 2. MBIC values, FBIC index and the nature of interaction between AOS and COS with AMP, CIP, CLI, TET and TMP against *S. aureus* wood 46.

	COS				AOS			
Antibiotics (ATB)	**ATB MBIC with COS (μg/ml)**	**COS MBIC with ATB (%)**	**FBIC**	**Interaction**	**ATB MBIC with AOS (μg/ml)**	**AOS MBIC with ATB (%)**	**FBIC**	**Interaction**
AMP	0.128	0.25	>4	Antagonist	0.032	0.25	1.42	Indifferent
CIP	0.5	0.25	1.03	Indifferent	0.5	0.25	1.01	Indifferent
CLI	0.0625	2	0.5	Synergy	0.125	1	0.73	Additive
TET	0.125	1	0.75	Additive	0.125	0.5	0.54	Additive
TMP	1	4	1.01	Indifferent	1	8	1.01	Indifferent

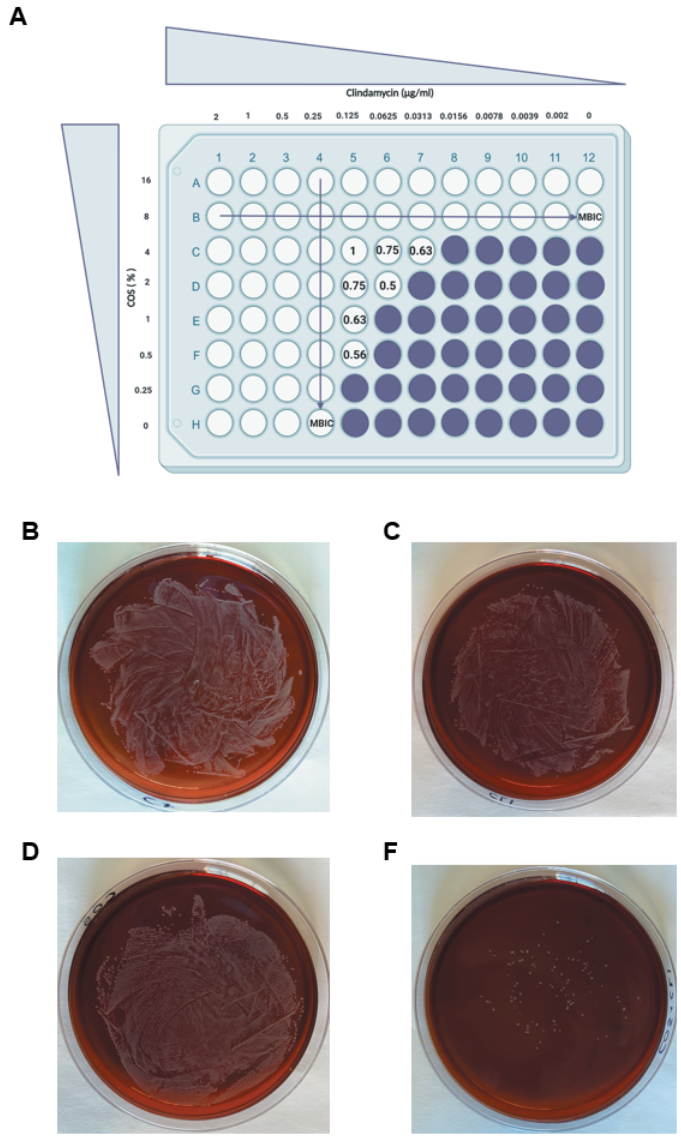

Figure 5. A simulated checkerboard assay for the combination of COS with CLI against biofilm formation of *S. aureus* and the subsequent growth of the synergistic combination on blood agar plates. As depicted in **Figure 5A** (38) , both COS and CLI were 2-fold serial diluted starting from 16 % COS (2 × MBIC) and 2 µg/mL CLI (8 × MBIC). Wells without color refer to full inhibition of *S. aureus* biofilm formation (≥ 90 % biofilm inhibition), while in purple wells the *S. aureus* biofilm formation was not fully inhibited. The FBIC values of the wells that showed synergistic or additive effect were calculated as described in the material and methods section using the following equation: ΣFBIC = FBIC A + FBIC B = (MBIC of drug A in the combination / MBIC of drug A alone) + (MBIC of drug B in the combination / MBIC of drug B alone) and depicted in the figure for each well separately. Partial synergy was identified when 2 % COS and 0.0625 µg/mL CLI were combined with a corresponding FBIC value of 0.5. This specific FBIC value is considered as the lowest FBIC index among the combi-

nation wells that had full biofilm formation inhibition. The far-right column represents the control of COS treatment (without CLI treatment) while the bottom row, in which COS is absent, represents the control of CLI treatment. Finally, the well on the bottom right corner, in which both COS and CLI are absent, is used as positive control for biofilm production. The combination of the antimicrobial agents that provoke the synergistic effect, 2 % COS with 0.0625 µg/mL CLI were grown on blood agar plates for 24h in order to determine whether the observed effect was attributed to their abilities to reduce the number of bacteria (**Figure 5F**). To identify the outcome of their interaction on bacterial growth, each agent was also grown separately, positive control which represents the absolute growth of bacteria without the presence of any treatment **(Figure 5B)**, CLI 0.0625 µg/mL (**Figure 5C**) and COS 2 % (**Figure 5D**). Finally, on a separate plate, the supernatant of the well without any intervention was grown, representing the positive control.

4. Discussion

In the present study, the anti-growth and anti-biofilm activities of AOS and COS against two pathogenic bacterial strains, GBS V and *S. aureus* wood 46, were evaluated. Furthermore, their combination with different antibiotics was tested to determine whether NDOs could enhance the function of antibiotics.

The antibacterial data revealed that AOS induce a strong inhibitory effect on the growth of GBS V, even at a low concentration (AOS 1%). One possible interpretation of such an effect is the anionic nature of AOS. Craft et al. showed that sialylated HMOs, which are negatively charged at homeostatic pH due to the sialic acid residues, exert antimicrobial activity against GBS III and Ia strains (39). In addition, the same group presented in another study that neutral fucosylated HMOs (2'-FL) did not have any substantial activity (40). Despite the fact that the negative charge is assumed to play a substantial role in the antimicrobial abilities of AOS, further investigations are needed to confirm this mechanism of action. AOS significantly reduced the growth of *S. aureus* wood 46, and therefore may act in a strain-dependent manner. Interestingly, a depolymerized product of alginate (a mannuronic acid derivative) demonstrated an inhibition and high inhibitory activity against *S. aureus* (41). The differences in the anti-pathogenic effects of AOS might be related to the match or mismatch between the structural features of AOS (negatively charged) and the strain-specific bacterial target structures.

While COS increased the growth of both bacterial strains, this increase was only significant in the case of *S. aureus* wood 46. Concerning the observed bacterial growth, it can be hypothesized that instead of exerting antimicrobial effects, COS was utilized as a beneficial source for the growth and survival of GBS and *S. aureus*. These results concur with an earlier study (42), in which chitosan exhibit the capacity to stimulate growth. This could be related to the positively charged amino groups of chitosan, which bind to surface components and cell debris instead of cell surfaces of the related pathogen (42). Moreover, it is possible that GBS could reproduce and utilize degraded chitosan as the sole carbon source to benefit their growth (42). These observations were in contrast with previous reports indicating that the antimicrobial activities of COS might relate to the interaction between the positively-charged COS and the negatively-charged membrane residues (e.g.

carbohydrate, proteins, lipids) (43–47). This effect can lead to cytoplasmic leakage and subsequently to cell death (48). For example, Benhabiles et al. (44), confirmed the inhibitory effects of N-acetyl COS (NAc-COS) and COS on the growth of *S. aureus* ATCC 25923 and *S. aureus* ATCC 43300. COS with a higher molecular weight (MW) (MW≥10 kDa) are more effective in inhibition of different microorganisms, such as *S. aureus*, compared to fractions with lower MW (45). These discrepancies might be attributable to methodological and experimental differences, such as bacterial strains and structural characteristics of COS. In this regard, it has previously been shown that the antibacterial effect of COS is greatly dependent on their degree of polymerization or their MW (49).

The potentiation of antibiotic activity in the therapy of multidrug-resistant organisms is a major goal of an anti-infective cure. In the present study, we investigated the ability of AOS to potentiate the activity of conventional antibiotics against the GBS V strain. AOS was selected since it exhibited the capacity to inhibit GBS V. Results obtained from the antibiotic sensitization assay indicated that AOS (2 % and 4 %) sensitizes GBS V to TMP by significantly decreasing the effective TMP concentration to observe a similar effect as obtained with more than 8 times higher TMP concentration. Although sensitization of GBS V occurred only in the case of TMP, 4 % AOS decreased the lowest effective concentration of CLI up to 4-fold (from 0.125 µg/mL to 0.0313 µg/mL CLI). Overall, a downward trend is observed in the growth of GBS V from all the combinations tested, however, this trend is mostly attributed to the effectivity of AOS starting from 8 % AOS with the most potent anti-growth ability observed with 4% and 2% AOS. Therefore, these insights into the effects of AOS might provide new opportunities to develop treatments of GBS-associated infections for which effective treatment is currently extremely limited.

Biofilms are one of the most challenging resistant mechanisms of bacteria that secrete various enzymes and virulence factors (50). Different mechanisms related to the inhibition of biofilm formation are modification of cell-surface charge, inhibition of bacterial growth, and prevention of microbial adhesion (51).

In our study, AOS significantly reduced the biofilm formation of both GBS V (4, 8 and 16 % AOS) and *S. aureus* wood 46 (8 and 16 % AOS). So far, anti-biofilm activities of AOS were only identified against gram-negative bacteria such as *P. aeruginosa* (19,52,53). To the best of our knowledge, this is the first study indicating the anti-biofilm effects of AOS against gram-positive bacteria. We hypothesized that the negative charges of AOS interact with positively charged components of the biofilm matrix. Indeed, concerning staphylococci biofilms, one of the most important positively charged polymers is called polysaccharide intercellular adhesin (PIA). PIA is involved in at least the majority of the staphylococcal biofilm-associated infections and constitutes the main molecule responsible for intercellular adhesion. The cationic PIA polymer (at neutral or basic pH) interacts with the negatively charged bacterial cell surface (e.g. with negatively charged teichoic acids) through multivalent electrostatic interactions (54). Since PIA plays such a significant role in

staphylococci biofilm formation, its inhibition by AOS could lead to obstructing the adhesive role of PIA and consequently inhibiting biofilm formation. There is limited information available related to the formation of streptococci biofilms, such as the composition of the GBS biofilm matrix. However, recent studies showed that this type of biofilm is mainly composed of proteins and eDNA, while polysaccharides represent a minor proportion (55,56). The anti-biofilm effect of AOS against GBS V can be attributed to the disruption of the intramolecular interactions in EPS that might occur due to the negative charge of AOS. These assumptions are in line with the results found by Taylor et al., who reported that alginates can disrupt intramolecular interaction in EPS (e.g. mucus), and competitively inhibit the interpolymer cross-links, weakening the biofilm structures (57). Anti-biofilm potential of AOS on the biofilms of GBS has not been identified so far. Therefore, further research is needed to identify the underlying mechanisms.

In contrast with AOS, the effect of COS on biofilm formation differs among the two bacterial species. COS treatment was ineffective against biofilm formation of GBS V, which is in line with the antibacterial data of COS in this study. On the other hand, there was a strong anti-biofilm inhibitory effect of different COS concentration (2, 4, 8 and 16 % COS) against *S. aureus* wood 46. Interestingly, the strong effect of COS against the biofilm formation of *S. aureus* wood 46 is in contrast with the observed (non-existing) antibacterial effect, since a significant increase in bacterial growth was observed. This contradiction might be attributed to the interference of COS with the compartments of the biofilm matrix that leads to the inhibition of biofilm formation, instead of adherence to the bacterial surface that has been previously proved to cause growth reduction (58). Our results supported the finding that chitosan displays an anti-biofilm activity against *S. aureus* strains of bovine origin (59). Additionally, a significant inhibitory effect of LMW chitosan on *S. aureus* V329 biofilm formation was demonstrated (58). Anti-biofilm properties of COS most likely rely on the polycationic nature due to its protonated amino groups, which interact electrostatically with the negatively charged biofilm components (e.g. proteins, eDNA) (60). Hence, this electrostatic interaction may inhibit the formation of biofilm (61). The observed inhibitory effect of COS on biofilm formation of *S. aureus* wood 46 in the current study can be attributed to the prevention of biofilm formation rather than on the destruction of preformed biofilms, since no changes were observed when COS was added for 24 h after formation of established biofilms (Supplementary figure 2A). Therefore, it can be hypothesized that COS interferes biofilm formation and development in early stages.

In summary, based on the anti-biofilm effects displayed by AOS and COS against *S. aureus* wood 46, a proposed mechanism of action is schematized in **Figure 6**. In comparison with the untreated bacteria that are able to form a mature biofilm, biofilm formation with treatment of AOS and COS is inhibited. This inhibitory anti-biofilm formation might be attributed to the charge of those two NDOs. AOS might electrostatically interact with the positively charged PIA while COS might interact with the negatively charged proteins and the eDNA of the extracellular matrix.

In support of this assumption: neutral fructo-oligosaccharides did not exert any anti-biofilm activity against *S. aureus* wood 46 (Supplementary figure 2B).

The development of bacterial resistance is one of the major concerns nowadays that affects the efficacy of several antibiotics for the treatment of severe infections. Combination therapy is considered as an effective approach to increase the potency of existing antibiotics and thereby combat antimicrobial resistance.

First, a checkerboard biofilm formation assay was performed testing the nature of the interactions of AOS and COS with five common-used antibiotics against *S. aureus* wood 46. The combination of COS and AOS with several antibiotics was investigated before, however, these studies was mainly focused on the reduction of bacterial growth rather than on the inhibition of biofilm formation (23,59,62,63). In the present study, the combination of COS with CLI and TET (targeting ribosomal protein 50S and 30S subunits respectively) showed synergistic and additive activity against *S. aureus* wood 46 biofilm formation, respectively. Additive interaction also occurred when AOS was combined with both antibiotics. COS can inhibit the biofilm formation through ionic interactions (60), therefore it can enhance the accumulation of CLI into the biofilm and inhibition of protein synthesis, showing a synergistic effect. Since CLI inhibits bacterial protein synthesis by binding to the 50S subunit of the bacterial rRNA inside the cell, it is expected that it can also reduce exoprotein production in *S. aureus* biofilms. Hu et al. (64) studied the effects of sub-inhibitory CLI on the production of *S. aureus* exoproteins and demonstrated that subinhibitory concentrations of CLI considerably decreases the *S. aureus* biofilm exoprotein. The additive effect observed by the interaction of AOS and COS with TET might be attributed to a similar mechanism of action, as TET also inhibits protein synthesis, although binding to the 30S subunit.

Interestingly, two bactericidal antibiotics (AMP and CIP) used in this study, showed indifferent or antagonistic effects, in combination with AOS and COS. Yang et al. (65) demonstrated that two bactericidal antibiotics (vancomycin and CIP) were tested on *Staphylococcus epidermidis* biofilms and found that the combinational treatment with these 2 antibiotics can reduce the efficacy of these individual treatments. These observations might be related to the formation of persister cells induced by stress from antibiotics (66). Moreover, the antagonistic effect deriving from the combination of COS with AMP can be partly explained by the fact that both agents target extracellular cell compartments. So, the positively charged group of COS can bind to the negatively charged compartments of the cell membrane, leading to the death of bacteria (48), while AMP inhibits cell wall synthesis (67), leading to competition among the two agents.

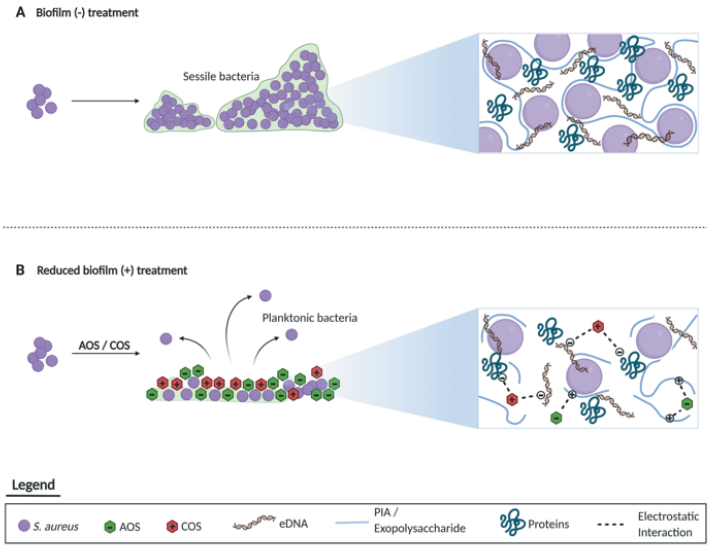

Figure 6. Representation of the proposed mechanism underlying the inhibitory effect of AOS and COS on biofilm formation of *S. aureus*. Concerning the untreated biofilm, a mature biofilm is formed by *S. aureus* in the absence of any treatment mainly composed of PIA, eDNA and proteins (**Figure 6A**). On the other hand, administration of AOS and COS may inhibit the formation of biofilm through the electrostatic interaction of oligosaccharides with the charged components of the extracellular matrix (**Figure 6B**). Created with BioRender.com.

5. Conclusion

In the present study, we have shown that AOS and COS modulate both bacterial growth and biofilm production of GBS V and *S. aureus* wood 46, respectively. In addition to the observed anti-growth and anti-biofilm properties of both NDOs, the anti-biofilm and anti-growth potential in combination with different antibiotics was evaluated. The synergistic effect of COS with CLI against *S. aureus* and the ability of AOS to sensitize GBS V to TMP were the most promising results offering new perspectives to help combat antimicrobial resistance. Given the increasing need for antimicrobial alternatives and the capability of NDOs to serve as antibacterial agents, future efforts should focus on assessing the antimicrobial effects of AOS and COS against additional species of both gram-positive and gram-negative pathogens and evaluating combination therapy with different antibiotics. Moreover, investigating the mechanisms underlying the antimicrobial, and anti-biofilm capacity of NDOs, as well as the interaction with antibiotics is necessary to further establish the therapeutic potential of NDOs.

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Author Contributions

Conceptualization MA, GF, RP, JP and SB; methodology, MA, GI; software, MA, GI; validation, MA, GI and SB; formal analysis, MA; investigation, MA, GI; resources, JP, GF; data curation, MA, GI; writing—original draft preparation, MA, GI; writing—review and editing, JP, SB, GF and RP; visualization, MA, GI; supervision, GF, SB; project administration, GF, RP, JP, SB; funding acquisition, GF, SB and RP. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

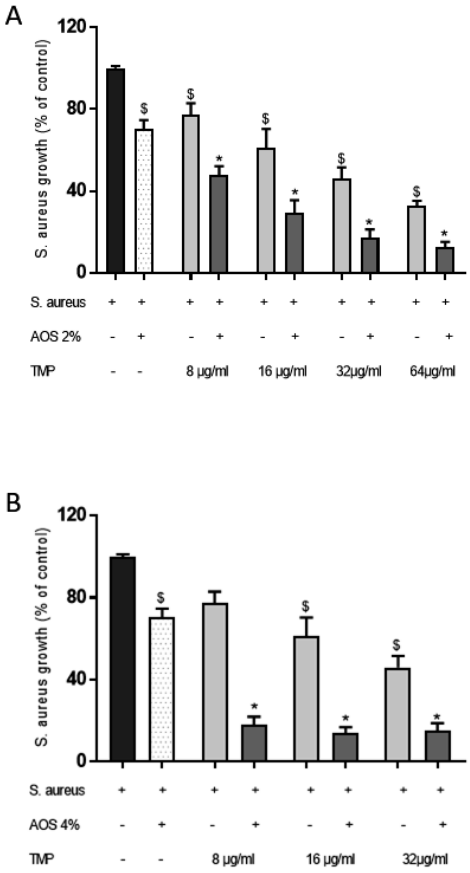
1. Lin AE, Autran CA, Szyszka A, Escajadillo T, Huang M, Godula K, et al. Human milk oligosaccharides inhibit growth of group B *Streptococcus*. J Biol Chem [Internet]. 2017;292(27):11243–9. Available from: http://dx.doi.org/10.1074/jbc.M117.789974
2. Tong SYC, Davis JS, Eichenberger E, Holland TL, Fowler VG. Staphylococcus aureus infections: Epidemiology, pathophysiology, clinical manifestations, and management. Clin Microbiol Rev. 2015;28(3):603–61.
3. Rosini R, Margarit I. Biofilm formation by *Streptococcus* agalactiae: Influence of environmental conditions and implicated virulence factor. Front Cell Infect Microbiol. 2015;5(FEB):2013–6.
4. Moormeier DE, Bayles KW. Staphylococcus aureus biofilm: a complex developmental organism. Mol Microbiol. 2017;104(3):365–76.
5. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: A common cause of persistent infections. Science (80-). 1999;284(5418):1318–22.
6. Jamal M, Ahmad W, Andleeb S, Jalil F, Imran M, Nawaz MA, et al. Bacterial biofilm and associated infections. Vol. 81, Journal of the Chinese Medical Association. Elsevier Ltd; 2018. p. 7–11.
7. Melin P. Neonatal group B streptococcal disease: from pathogenesis to preventive strategies. Clin Microbiol Infect. 2011;17:1294–303.
8. Kolter J, Henneke P. Codevelopment of microbiota and innate immunity and the risk for group B streptococcal disease. Vol. 8, Frontiers in Immunology. Frontiers Media S.A.; 2017. p. 1.
9. Brandt SL, Putnam NE, Cassat JE, Serezani CH. Innate Immunity to Staphylococcus aureus : Evolving Paradigms in Soft Tissue and Invasive Infections . J Immunol. 2018;200(12):3871–80.
10. Boakes E, Woods A, Johnson N, Kadoglou N. Breast Infection: A Review of Diagnosis and Management Practices. Eur J Breast Heal. 2018;(12):136–43.
11. Lindberg E, Adlerberth I, Hesselmar B, Saalman R, Strannegård IL, Åberg N, et al. High Rate of Transfer of Staphylococcus aureus from Parental Skin to Infant Gut Flora. J Clin Microbiol. 2004 Feb;42(2):530–4.
12. Craft KM, Gaddy JA, Townsend SD. Human Milk Oligosaccharides (HMOs) Sensitize Group B *Streptococcus* to Clindamycin, Erythromycin, Gentamicin, and Minocycline on a Strain Specific Basis. ACS Chem Biol. 2018;13(8):2020–6.
13. Wright GD. Antibiotic Adjuvants: Rescuing Antibiotics from Resistance. Trends Microbiol. 2016;24(11):862–71.
14. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: An emergent form of bacterial life. Vol. 14, Nature Reviews Microbiology. Nature Publishing Group; 2016. p. 563–75.
15. Reffuveille F, Josse J, Vallé Q, Mongaret C, Gangloff SC. Staphylococcus aureus biofilms and their impact on the medical field. Tech. 2017 Mar;
16. Bjarnsholt T, Ciofu O, Molin S, Givskov M, Høiby N. Applying insights from biofilm biology to drug development-can a new approach be developed? Vol. 12, Nature Reviews Drug Discovery. Nature Publishing Group; 2013. p. 791–808.
17. Asadpoor M, Ithakisiou G.N PAJH, Pieters R, Folkerts G, Braber and S. Non-Digestible Oligosaccharides and Short Chain Fatty Acids as Therapeutic Targets against Enterotoxin-Producing Bacteria and Their Toxins. Toxins (Basel). 2021;13(3):175.
18. Asadpoor M, Peeters C, Henricks PAJ, Varasteh S, Pieters RJ, Folkerts G, et al. Anti-pathogenic functions of non-digestible oligosaccharides in vitro. Nutrients. 2020 Jun;12(6):1789.
19. Powell LC, Pritchard MF, Ferguson EL, Powell KA, Patel SU, Rye PD, et al. Targeted disruption of the extracellular polymeric network of Pseudomonas aeruginosa biofilms by alginate oligosaccharides. npj Biofilms Microbiomes. 2018 Dec;4(1):1–10.
20. Ackerman DL, Craft KM, Doster RS, Weitkamp JH, Aronoff DM, Gaddy JA, et al. Antimicrobial and Antibiofilm Activity of Human Milk Oligosaccharides against *Streptococcus* agalactiae, Staphylococcus aureus, and Acinetobacter baumannii. ACS Infect Dis. 2018;4(3):315–24.

21. Craft KM, Thomas HC, Townsend SD. Interrogation of Human Milk Oligosaccharide Fucosylation Patterns for Antimicrobial and Antibiofilm Trends in Group B *Streptococcus*. ACS Infect Dis. 2018 Dec;4(12):1755–65.
22. Lu Y, Slomberg DL, Schoenfisch MH. Nitric oxide-releasing chitosan oligosaccharides as antibacterial agents. Biomaterials. 2014 Feb;35(5):1716–24.
23. Khan S, Tøndervik A, Sletta H, Klinkenberg G, Emanuel C, Onsøyen E, et al. Overcoming drug resistance with alginate oligosaccharides able to potentiate the action of selected antibiotics. Velindre NHS Trust. 2012;
24. Vor L de, Dijk B van, Kessel KPM van, Kavanaugh JS, Haas CJC de, Aerts PC, et al. Human monoclonal antibodies against Staphylococcus aureus surface antigens recognize in vitro biofilm and in vivo implant infections. bioRxiv. 2021 Feb;2021.02.09.429966.
25. Sheen TR, Jimenez A, Wang N-Y, Banerjee A, Sorge NM van, Doran KS. Serine-Rich Repeat Proteins and Pili Promote *Streptococcus* agalactiae Colonization of the Vaginal Tract. J Bacteriol. 2011 Dec;193(24):6834.
26. Asadpoor M, Varasteh S, Pieters R, Folkerts G, Braber S. Differential effects of oligosaccharides on the effectiveness of ampicillin against Escherichia coli in vitro. PharmaNutrition. 2021;(February).
27. Pinna A, Donadu MG, Usai D, Dore S, D'Amico-Ricci G, Boscia F, et al. In vitro antimicrobial activity of a new ophthalmic solution containing povidone-iodine 0.6% (IODIM®). Acta Ophthalmol. 2020;98(2):e178–80.
28. Kothary V, Doster RS, Rogers LM, Kirk LA, Boyd KL, Romano-Keeler J, et al. Group B *streptococcus* induces neutrophil recruitment to gestational tissues and elaboration of extracellular traps and nutritional immunity. Front Cell Infect Microbiol. 2017;7(FEB):1–13.
29. Zheng W, Sun W, Simeonov A. Drug repurposing screens and synergistic drug-combinations for infectious diseases. Br J Pharmacol. 2018 Jan;175(2):181–91.
30. Sun W, Weingarten RA, Xu M, Southall N, Dai S, Shinn P, et al. Rapid antimicrobial susceptibility test for identification of new therapeutics and drug combinations against multidrug-resistant bacteria. Emerg Microbes Infect. 2016 Nov;5(11):116.
31. Marks LR, Clementi EA, Hakansson AP. Sensitization of Staphylococcus aureus to Methicillin and Other Antibiotics In Vitro and In Vivo in the Presence of HAMLET. PLoS One. 2013;8(5):1–11.
32. Ruppen C, Hemphill A, Sendi P. *In vitro* activity of gentamicin as an adjunct to penicillin against biofilm group B *Streptococcus*. J Antimicrob Chemother. 2017 Feb;72(2):444–7.
33. Kang J, Jin W, Wang J, Sun Y, Wu X, Liu L. Antibacterial and anti-biofilm activities of peppermint essential oil against Staphylococcus aureus. LWT. 2019 Mar;101:639–45.
34. Mataraci E, Dosler S. In vitro activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin-resistant Staphylococcus aureus biofilms. Antimicrob Agents Chemother. 2012;56(12):6366–71.
35. Abdi Ali A, Shafiei M, Shahcheraghi F, Saboora A, Ghazanfari T. The Study of Synergistic Effects of n.butanolic Cyclamen coum Extract and Ciprofloxacin on inhibition of Pseudomonas aeruginosa biofilm formation. Biol J Microorg. 2015 Jan;3(12):25–32.
36. Feldman M, Smoum R, Mechoulam R, Steinberg D. Potential combinations of endocannabinoid/endocannabinoid-like compounds and antibiotics against methicillin-resistant Staphylococcus aureus. PLoS One. 2020 Apr;15(4):e0231583.
37. Ramezani K, Akbari A, Ahya ;, Ali A. Study of antimicrobial effects of several antibiotics and iron oxide nanoparticles on biofilm producing pseudomonas aeruginosa. 2017;4(1):37–43.
38. Lorian V. Antimicrobial Combinations, in Antibiotics In Laboratory Medicine. 5th ed. Philadelphia, Pa, USA: Lippincott Williams and Wilkins, 365–440.; 2005.
39. Craft KM, Thomas HC, Townsend SD. Sialylated variants of lacto-: N -tetraose exhibit antimicrobial activity against Group B *Streptococcus*. Org Biomol Chem. 2019;17(7):1893–900.
40. Craft KM, Townsend SD. 1-Amino-2′-fucosyllactose inhibits biofilm formation by *Streptococcus* agalactiae. J Antibiot (Tokyo). 2019;72(6):507–12.
41. Hu X, Jiang X, Gong J, Hwang H, Liu Y, Guan H. Antibacterial activity of lyase-depolymerized products of alginate. J Appl Phycol. 2005 Jan;17(1):57–60.

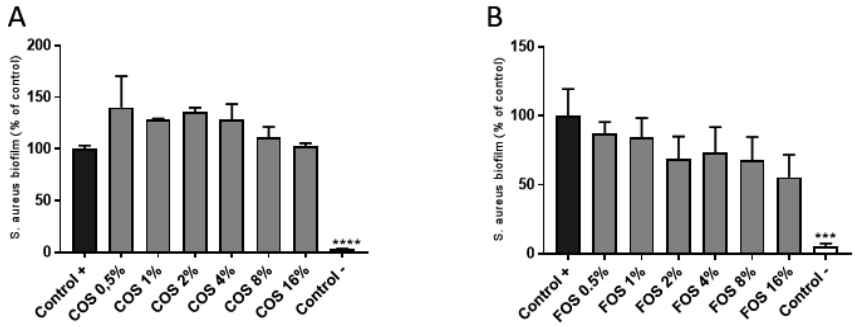
42. Yildirim-Aksoy M, Beck BH, Zhang D. Examining the interplay between *Streptococcus* agalactiae, the biopolymer chitin and its derivative. Microbiologyopen. 2019 May;8(5).
43. Moon JS, Kim HK, Koo HC, Joo YS, Nam HM, Park YH, et al. The antibacterial and immunostimulative effect of chitosan-oligosaccharides against infection by Staphylococcus aureus isolated from bovine mastitis. Appl Microbiol Biotechnol. 2007 Jul;75(5):989–98.
44. Benhabiles MS, Salah R, Lounici H, Drouiche N, Goosen MFA, Mameri N. Food Hydrocolloids Antibacterial activity of chitin , chitosan and its oligomers prepared from shrimp shell waste. Food Hydrocoll [Internet]. 2012;29(1):48–56. Available from: http://dx.doi.org/10.1016/j.foodhyd.2012.02.013
45. Jeon YJ, Park PJ, Kim SK. Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr Polym. 2001 Jan;44(1):71–6.
46. Zheng LY, Zhu JF. Study on antimicrobial activity of chitosan with different molecular weights. Carbohydr Polym. 2003 Dec;54(4):527–30.
47. Lin S-B, Chen S-H, Peng K-C. Preparation of antibacterial chito-oligosaccharide by altering the degree of deacetylation of β-chitosan in a *Trichoderma harzianum* chitinase-hydrolysing process. J Sci Food Agric. 2009 Jan;89(2):238–44.
48. Liu H, Du Y, Wang X, Sun L. Chitosan kills bacteria through cell membrane damage. Int J Food Microbiol. 2004 Sep;95(2):147–55.
49. Jeon YJ, Kim SK. Production of chitooligosaccharides using an ultrafiltration membrane reactor and their antibacterial activity. Carbohydr Polym. 2000 Feb;41(2):133–41.
50. Graf AC, Leonard A, Schäuble M, Rieckmann LM, Hoyer J, Maass S, et al. Virulence factors produced by staphylococcus aureus biofilms have a moonlighting function contributing to biofilm integrity. Mol Cell Proteomics. 2019 Jun;18(6):1036–53.
51. Roy R, Tiwari M, Donelli G, Tiwari V. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence. 2018;9.
52. He X, Hwang H, Aker WG, Wang P, Lin Y, Jiang X, et al. Synergistic combination of marine oligosaccharides and azithromycin against Pseudomonas aeruginosa. Microbiol Res [Internet]. 2014;169(9–10):759–67. Available from: http://dx.doi.org/10.1016/j.micres.2014.01.001
53. Powell LC, Sowedan A, Khan S, Wright CJ, Hawkins K, Onsøyen E, et al. The effect of alginate oligosaccharides on the mechanical properties of gram-negative biofilms. Biofouling. 2013 Apr;29(4):413–21.
54. Arciola CR, Campoccia D, Ravaioli S, Montanaro L. Polysaccharide intercellular adhesin in biofilm: Structural and regulatory aspects. Vol. 5, Frontiers in Cellular and Infection Microbiology. Frontiers Research Foundation; 2015.
55. D'Urzo N, Martinelli M, Pezzicoli A, De Cesare V, Pinto V, Margarit I, et al. Acidic pH strongly enhances in vitro biofilm formation by a subset of hypervirulent ST-17 *Streptococcus* agalactiae strains. Appl Environ Microbiol. 2014;80(7):2176–85.
56. Alvim DCSS, Ferreira AFM, Leal MA, Oliveira LMA, Botelho AMN, Botelho ACN, et al. Biofilm production and distribution of pilus variants among *Streptococcus* agalactiae isolated from human and animal sources. Biofouling. 2019 Sep;35(8):938–44.
57. Taylor C, Draget KI. Oligosaccharides As Modulators of Rheology in Complex Mucous Systems. 2011;3084–90.
58. Felipe V, Breser ML, Bohl LP, Rodrigues da Silva E, Morgante CA, Correa SG, et al. Chitosan disrupts biofilm formation and promotes biofilm eradication in Staphylococcus species isolated from bovine mastitis. Int J Biol Macromol. 2019 Apr;126:60–7.
59. Asli A, Brouillette E, Ster C, Ghinet MG, Brzezinski R, Lacasse P, et al. Antibiofilm and antibacterial effects of specific chitosan molecules on Staphylococcus aureus isolates associated with bovine mastitis. Becker K, editor. PLoS One. 2017 May;12(5):e0176988.
60. Khan F, Pham DTN, Oloketuyi SF, Manivasagan P, Oh J, Kim YM. Chitosan and their derivatives: Antibiofilm drugs against pathogenic bacteria. Vol. 185, Colloids and Surfaces B: Biointerfaces. Elsevier B.V.; 2020. p. 110627.
61. Jiang F, Deng Y, Yeh CK, Sun Y. Quaternized chitosans bind onto preexisting biofilms and eradicate pre-attached microorganisms. J Mater Chem B. 2014 Dec;2(48):8518–27.

62. Tin S, Sing Lim C, Sakharkar M, Ramaji Sakharkar K. Synergistic Combinations of Chitosans and Antibiotics in Staphylococcus aureus. Lett Drug Des Discov. 2010 Jan;7:31–5.
63. Kim J-H, Yu D, Eom S-H, Kim S-H, Oh J, Jung W-K, et al. Synergistic Antibacterial Effects of Chitosan-Caffeic Acid Conjugate against Antibiotic-Resistant Acne-Related Bacteria. Mar Drugs. 2017;15:6.
64. Hu, Ramezanpour, Hayes, Liu, Psaltis, Wormald, et al. Sub-Inhibitory Clindamycin and Azithromycin reduce S. aureus Exoprotein Induced Toxicity, Inflammation, Barrier Disruption and Invasion. J Clin Med. 2019 Oct;8(10):1617.
65. Yang S, Hay ID, Cameron DR, Speir M, Cui B, Su F, et al. Antibiotic regimen based on population analysis of residing persister cells eradicates Staphylococcus epidermidis biofilms. Sci Rep. 2015 Dec;5.
66. Dörr T, Vulić M, Lewis K. Ciprofloxacin causes persister formation by inducing the TisB toxin in Escherichia coli. PLoS Biol. 2010;8(2):29–35.
67. Epand RM, Walker C, Epand RF, Magarvey NA. Molecular mechanisms of membrane targeting antibiotics. Biochim Biophys Acta - Biomembr. 2016 May;1858(5):980–7.

Supplementary Figures



Supplementary Figure 1. Effective combination of 2% and 4% AOS and TMP on the growth of GBS. To examine whether 2% and 4% AOS have the feasibility to sensitize GBS to TMP, a sensitization assay was performed as described in the material and method section. AOS (2%) were combined with different concentrations of TMP (**Supplementary figure 1A**) and AOS (4%) were combined with different concentrations of TMP (**Supplementary figure 1B**). Star (*) is representing a significant reduction ($P < 0.05$) of the combinational treatments comparing with corresponding antimicrobial agents (AOS and antibiotics) and positive control. Dollar ($) is representing a significant reduction ($P < 0.05$) of AOS or TMP groups comparing with positive control. Positive control represents the percentage of the absolute growth of bacteria (100 % growth) without the presence of any treatment. The results are expressed as the percentage of bacterial growth as mean ± SEM of three independent experiments each performed in a minimum of 3 replicates. Two-way ANOVA test was used for statistical analysis.



Supplementary Figure 2. Preventive effect of biofilm formation by FOS and anti-biofilm activity of COS against *S. aureus*. For the biofilm formation assay, six different concentrations (0.5-16 %) of COS and FOS were tested for 24 h after formation of established biofilms (**Supplementary figure 2A**) and 24 h before formation of established biofilm (**Supplementary figure 2B**), respectively, as described in the material and methods section. Control (-) represents the negative control (uninoculated culture media without NDO treatment) and control (+) represents full-formed biofilms without any additional treatment. Results are expressed as the percentage of control (relative to positive control) as mean ± SEM of three independent experiments each performed in triplicate. Statistical differences *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) and ****($P < 0.0001$) compared to positive control were obtained using one-way ANOVA test.

Chapter 5

Non-Digestible Oligosaccharides and Short Chain Fatty Acids as Therapeutic Targets against Enterotoxin-Producing Bacteria and Their Toxins

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Abstract

Enterotoxin-producing bacteria (EPB) have developed multiple mechanisms to disrupt gut homeostasis, and provoke various pathologies. A major part of bacterial cytotoxicity is attributed to the secretion of virulence factors, including enterotoxins. Depending on their structure and mode of action, enterotoxins intrude the intestinal epithelium causing long-term consequences such as hemorrhagic colitis. Multiple non-digestible oligosaccharides (NDOs), and short chain fatty acids (SCFA), as their metabolites produced by the gut microbiota, interact with enteropathogens and their toxins, which may result in the inhibition of the bacterial pathogenicity. NDOs characterized by diverse structural characteristics, block the pathogenicity of EPB either directly, by inhibiting bacterial adherence and growth, or biofilm formation or indirectly, by promoting gut microbiota. Apart from these abilities, NDOs and SCFA can interact with enterotoxins and reduce their cytotoxicity. These anti-virulent effects mostly rely on their ability to mimic the structure of toxin receptors and thus inhibiting toxin adherence to host cells. This review focuses on the strategies of EPB and related enterotoxins to impair host cell immunity, discusses the anti-pathogenic properties of NDOs and SCFA on EPB functions and provides insight into the potential use of NDOs and SCFA as effective agents to fight against enterotoxin.

Keywords: enterotoxins; enteropathogenic bacteria; oligosaccharides; short chain fatty acids.

1. Introduction

Currently, impairment of the gastrointestinal tract caused by the activity of bacterial enteropathogens is one of the biggest issues affecting human health and food safety (1). Because of a growing concern on the relationship between toxigenic bacteria and intestinal associated diseases, research is required to define conditions and minimize the levels of their toxicity. Dietary carbohydrates, especially non-digestible oligosaccharides (NDOs) and short chain fatty acids (SCFA) as their metabolites produced by the gut microbiota, are known to reduce the toxic potential of bacterial enteropathogens in multiple stages of their pathogenicity (2). Furthermore, the NDOs possess important physiological and physicochemical properties and serve as dietary fibers and prebiotics. Additionally, NDOs of various origins have been used extensively as immunostimulators, animal feed, agrochemicals, cosmetics and for drug delivery (3).

The human gut microbiota harbors a diverse community of commensal bacteria with a vast biosynthetic capacity. The role of the microbiota and its residents is essential for the host, since it regulates multiple functions, including immune system development, nutrient processing and prevention of pathogen colonization (4). The intestinal gut microbiota is directly exposed to the external environment, and therefore highly susceptible to pathogenic invasion and colonization (5). Intestinal epithelial cells can be targeted by various pathogenic bacteria and consequently by their virulence factors, such as, toxins. More specifically, toxins secreted by bacteria that selectively interact with intestinal cells are called enterotoxins. Following different modes of actions, including pore formation, increase in permeability of the intestinal epithelium and alterations in cell homeostasis, enterotoxins can cause different gastrointestinal diseases such as pseudomembranous colitis (6). Among the major pathogenic bacteria that secrete highly toxic proteins, enterotoxin-producing *Bacillus cereus*, *Clostridium difficile*, *Clostridium perfringens*, *Escherichia coli*, *Staphylococcus aureus* and *Vibrio cholerae* are the most prominent. Therefore, a clear understanding of key features of toxicogenic bacteria as well as their virulent products is required for the development and selection of optimal treatments.

To date, antibiotics are the most promising therapy for diseases related to enterotoxin producing bacteria (EPB), however, exponential use and misuse of antibiotics have led to loss of their efficacy and antimicrobial resistance (7) Several mechanisms of antibiotic resistant pathogenic bacteria render these antimicrobials inactive and prolong their survival and pathogenicity by, for example, biofilm formation. Since many infections remain untreated, antibiotic resistance in bacterial pathogens is one of the great challenges in the developed and developing world with immense clinical and economic impacts (8). Therefore, new strategies to resolve this escalating problem and diminish bacterial resistance associated infections are urgently needed. Over recent decades, there is a growing interest in NDOs as anti-pathogenic agents, since NDOs do not only maintain gut homeostasis, but can also exert microbiota-independent effects on intestinal epithelial and immune cells with minimum side effects (9)

NDOs obtained from natural sources or manufactured via enzymatic or chemical synthesis, can get fermented by the beneficial bacteria to release metabolic substrates and energy (10). Additionally, according to their key characteristics, such as, monosaccharide building blocks, degree of polymerization (DP), degree of acetylation (DA) and charge, they exhibit anti-pathogenic effects in multiple ways. Antimicrobial capabilities of NDOs are not limited against pathogenic bacteria, but also anti-virulent properties by blocking virulence factors, such as enterotoxins, have been described. Receptor mimicry mechanisms and stimulation or blocking of intracellular pathogenic mechanisms are some of the strategies that NDOs use to encounter such toxins. SCFA, as the end products of oligosaccharide fermentation induced by anaerobic intestinal microbiota, can induce similar anti-toxic effects, but through different mechanisms. Among diverse SCFA, acetate, propionate and butyrate have shown the most prominent anti- pathogenic effects (11).

This review aims to explore the current state of knowledge on the anti-virulence strategies of NDOs and SCFA against pathogenic bacteria and associated enterotoxins that target the intestinal epithelial layer. The review starts with describing structural characteristics and mode of action of the major virulent enterotoxins, including enterotoxins related to *B. cereus*, *C. difficile* and *C. perfringens*, *cholera toxin* (CT), heat-labile (LT) and heat-stable (ST) enterotoxins, *Shiga toxins* (Stxs) and staphylococcal enterotoxins (SEs). Thereafter, the main characteristics of the described NDOs are presented followed by a comprehensive overview of the anti-microbial functionalities of NDOs and SCFA against EPB and their enterotoxins. First, the anti-pathogenic properties of NDOs and SCFA against the EPB are specified "directly" by exerting anti-adhesive, anti-biofilm and anti-growth effects against EPB and "indirectly" by promoting the growth of beneficial bacteria that maintain gut homeostasis, mainly through SCFA production, which results in a reduction in final colonization and prefiltration of EPB. Second, the mechanisms of action (both direct and indirect) of NDOs and SCFA against each enterotoxin are discussed, which may open new avenues for NDOs and SCFA as effective agents to fight against enterotoxins.

2. Enterotoxin-Producing Bacteria and Related Enterotoxins

The pathogenicity of various bacteria on the human intestinal epithelium is associated with their ability to produce certain virulence factors, called enterotoxins. Enterotoxins tend to be produced by Gram-positive bacteria, however, some exceptions of Gram-negative bacteria, such as *E. coli* and *V. cholerae*, are also characterized as enterotoxin-producing bacteria. According to their structure and pathogenic characteristics, enterotoxins can cause different pathogenic effects, such as the disturbance of the cellular ionic balance due to membrane pore formation and overstimulation of the immune response. A detailed investigation of the characteristics and the cytotoxicity pathways of both enterotoxin-producing bacteria and enterotoxins can provide insights in potential therapeutic treatments.

2.1. *B. cereus* and Related Enterotoxins

2.1.1. Epidemiology of *B. cereus* and Related Enterotoxins

B. cereus, a Gram-positive microorganism, is a versatile, spore-forming and facultative anaerobe abundantly found in nature, most commonly isolated from soil, growing plants, as well as in food production environments. *B. cereus* is a motile bacterium with a wide growth temperature range (8–55 °C). Its ability to form spores render it highly resistant to harsh environmental conditions, such as low pH values, drought or radiation (12).

The spread from bacterium habitats to foods and therefore to humans can cause two types of foodborne disease: an intoxication (emetic form) and a toxico-infection (diarrheal form). At the diarrheal syndromes of *B. cereus* infections, three protein enterotoxins are implicated, the hemolytic hemolysin B (Hbl), the non-hemolytic enterotoxin (Nhe) and the cytotoxin K (CytK) (13,14). Even if associated symptoms in some cases can be mild and self-limited, like abdominal pain, watery diarrhea and nausea, lethal cases have also been reported (15).

2.1.2. Structure of Hbl, Nhe and CytK

Similar structure and functionality characterize Hbl and Nhe enterotoxins as each of them is composed of three different protein compartments, resulting in a membrane attacking complex that leads to lysis of target cells. More specifically, Hbl consists of L2, L1 and B proteins produced at an equivalent ratio, while Nhe is composed of NheA, NheB and NheC proteins produced at a ratio close to 10:10:1 (NheA: B: C). Hbl L2 shows sequence homology to NheA, Hbl L1 to NheB and Hbl B to NheC. For exhibiting their maximal enterotoxic activity, the synergistic function of all three proteins is necessary. Concerning Hbl, X-ray crystallography results revealed a long α-helical bundle and a small α/β head domain to the toxin structure. Based on the similarity of the Hb1 structure with the structure of *E. coli* hemolysin E (HlyE, ClyA, SheA), pore formation was suggested as a mode of action for Hbl. Nhe also belongs to the pore formation toxins, however, its structure is distinct from the structure of Hbl, while it also lacks hemolytic activity (16). CytK (34 kDa), or alternatively called hemolysin IV, is a single component, β-barrel channel-forming toxin (17). Two different forms of CytK have been investigated, CytK 1 and CytK 2. CytK 1 was found to be five-fold more toxic to human intestinal Caco-2 and Vero cells than CytK 2 is (16).

2.1.3. Pathogenicity of Hbl, Nhe and CytK

In their structure, all B. cereus-related enterotoxins contain secretory signal peptides, a fact that indicates their secretion by the general secretory (Sec) pathway (17). To date, for Hbl and Nhe, no cell membrane receptors have been identi-fied so far (17). However, some studies reported evidence of Nhe cellular activity which begins with the binding of NheC and NheB proteins. After binding, NheC and NheB oligomers get attached to the cell membranes to form prepores and finally NheA becomes associated with the NheB-C complex and penetrates the lipid bilayer leading to cell death (18). Based on these studies, pore formation relies on a stepwise, sequential binding of NheC, NheB and NheA and, Hbl

B, Hbl L1, and Hbl L2, respectively (19). According to the significant amino acid sequence similarity between Nhe and Hbl, in addition to the fact that crystal structure of Hbl B resembles the well-known cytolysin A, it is likely that Hbl, like Nhe, also belongs to the α pore-forming toxins (PFT) family (18). To achieve the optimal pore formation and the maximum cytotoxicity at the target cell surface, specific binding order as well as a specific concentration ration is needed from each enterotoxin separately.

CytK was recently described, therefore investigations about its pathogenic mechanism are still limited. However, *in vitro* studies have demonstrated its ability to form pores in the epithelial cells and therefore result in fluid release and necrosis due to epithelial cell destruction. Based on their ability to form pores in phospholipid membranes, it was believed that it is unlikely that receptors are absolutely necessary for binding and lysis by CytK (17). To conclude, the pathogenicity and relation of all three enterotoxins with diarrheal effects is based on their ability to damage the integrity of the plasma membrane of the small intestine by forming pores that allow influx of Ca^{2+}, Na^{+} and efflux of K^{+} and ATP and provoke cell death (Figure 1A).

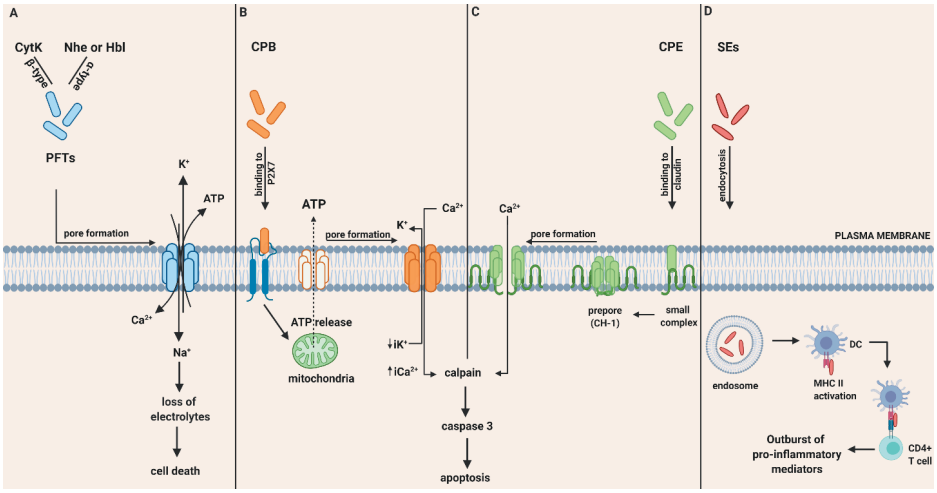


Figure 1. Intoxication pathways of hemolysin B (Hbl), non-hemolytic enterotoxin (Nhe), cytotoxin K (Cyt K), CPB, CPE and staphylococcal enterotoxins (SEs). (A) Proposed mechanism of B. cereus enterotoxins, Hbl, Nhe and Cyt K. Hbl, Nhe and CytK begin their cytotoxic pathway by forming cell membrane pores that lead to the influx of Ca^{2+} and Na^{+} and the efflux of K^{+} and ATP and thereby to the loss of electrolytes and cell death. (B) Intracellular action of *C. perfringens* beta-toxin (CPB). Once CPB binds to ATP-gated P2X7 receptor, ATP is released from target cells to ATP-release channel pannexin 1. Induced by ATP release, CPB is oligomerized and a pore is formed. Pore formation leads to increased influx of Ca^{2+} that triggers calpain activation and necroptosis. Furthermore, pore formation also results in loss of intracytoplasmic K^{+} (iK^{+}) that is associated with the activation of MAPK and JNK that are responsible for host cell survival and defense pathways. (C) Intracellular action of *C. perfringens* enterotoxin (CPE). CPE binds to its cellular receptor, claudin, and forms a small complex. Later on, six small CPE complexes oligomerized forming a prepore on the plasma membrane called CH-1. Assembly of β-hairpin loops into a β-barrel structure allows a cation-permeating pore insertion in the plasma membrane. The influx of Ca^{2+} stimulates calpain activity and thus the activation of caspase-3 and apoptosis. (D) SE-associated gastrointestinal (GI) inflammatory injury. Once they get endocytosed, SEs bind to MHC II class molecules and subsequently attract CD^{4+} T cells. Afterwards, an excessive production of pro inflammatory chemokines and cytokines is induced. Created with biorender.com (accessed on 28 May 2020).

2.2. *C. perfringens* and Related Enterotoxins

C. perfringens, a Gram-positive, spore-forming and anaerobic bacterium, which is one of the components of the normal gastrointestinal (GI) tract microbiota of both humans and animals. Except humans and animals, it can be also found in soil, food and sewage. The virulence of *C. perfringens* is attributed to the production of different toxins depending on the different strains. Its presence is associated with various histotoxic, enteric and enterotoxemic diseases due to this large production of enterotoxins, including traumatic gas gangrene, foodborne illnesses and enteritis necroticans (20).

C. perfringens strains are classified in five different types (A–E) according to the presence of encoding genes for alpha (α), beta (β), epsilon (ε) and iota (ι) toxins (20). Recently, this classification system was revised and expanded with two more bacterial strains, type F

strains, producing *C. perfringens* enterotoxin (CPE) and type G strains, producing necrotic enteritis B-like toxin (NetB) (21). In this review, we focused on *C. perfringens* beta toxin (CPB) and CPE enterotoxins provoking human intestinal diseases.

2.2.1. *C. perfringens* Beta Toxin (CPB)

Epidemiology of CPB

C. perfringens beta toxin (CPB) is the causative agent of foodborne necrotizing enterocolitis in humans, produced by type C strains. Additionally, termed as Pig-bel, necrotizing enterocolitis was historically more related to the Highland of Papua New Guinea and it was mainly caused by the ingestion of insufficiently cooked pork. Disease symptoms include serious bloody diarrhea, abdominal pain, distention and emesis (22).

Structure of CPB

CPB is expressed as a 336-amino-acid single polypeptide belonging to the β-PFT family. During CPB secretion, a 27-amino-acid signal sequence is removed, leading to the formation of the mature toxin (35 kDa). Purified CPB is highly sensitive to protease treatment and is thermolabile (23). As a member of the β-PFT family, CPB shares sequence homology with other toxins, including *C. perfringens* delta toxin (43% identity) and several *S. aureus* toxins, including alpha toxin (28% identity) (24).

Mode of Action of CPB

CPB is expressed as a prototoxin that includes a signal sequence of 27 amino acids, which is removed upon toxin secretion, leading to a mature protein. CPB selectively binds to the ATP-gated P2X7 receptor on the plasma membrane. This binding is related to ATP release from the target cell through the ATP Pannexin 1 channel. ATP release facilitates CPB to get oligomerized and form functional pores. Through these pores, the efflux of intracytoplasmic K^+ (iK^+) and the entry of Ca^{2+}, Na^+ and Cl– is allowed. On the other hand, influx of Ca^{2+} is associated with calpain activation and necroptosis (Figure 1B) (21).

2.2.2. *C. perfringens* Enterotoxin (CPE)

Epidemiology of CPE

CPE represents a very important cause of different human illnesses, including foodborne and non-foodborne GI diseases (21). CPE is mainly produced by type F CPE-positive strains, however, being produced by type C and D strains is also common (21). Specifically, CPE-positive type F strains are the main etiological factor of *C. perfringens* type F food poisoning (previously referred as *C. perfringens* type A food poisoning), which is ranked as the second most frequent foodborne illness in most developed countries. In the USA, around one million food poisoning cases are estimated each year due to *C. perfringens* infection (25). Symptoms like diarrhea and abdominal cramping caused by type F food poisoning have found to be highly associated with the presence of CPE and under predisposing conditions,

CPE can also be fatal. In addition to food poising, CPE-positive type F strains cause 5 to 15% of all non-foodborne human GI diseases, such as, antibiotic-associated diarrhea and sporadic diarrhea. Additionally, CPE-positive type C strains are also responsible for some incidences of enteritis necroticans, a form of inflammatory gut disorder, even if the beta toxin constitutes the primary role in the pathogenesis of this disease (26).

Structure of CPE

CPE is a pore forming two-domain protein composed of a single chain polypeptide of 319 amino acids (35 kDa). The C-terminal domain (C-CPE, residues 184–319) is the receptor-binding domain that recognizes and binds to claudins as the receptors of the toxin. The N-terminal domain is involved in oligomerization and pore formation that disrupt plasma membrane, leading to cell death. A specific region of the N-terminal domain (residues 80–106) termed as TM1, consists of amino acids that resemble the β-hairpin loops, which are known to mediate membrane insertion and pore formation of several bacterial pore-forming toxins. This ability of the TM1 region largely corresponds to an α helix located in the N-terminal domain of CPE. Consequently, the α helix likely unfolds into a β-hairpin loop during pore formation and membrane penetration (27).

Mode of Action of CPE

Pathogenicity of type F by the *C. perfringens* strain begins with the consumption of food, contaminated with vegetative cells. In severe cases when the ingested food is highly infected, bacteria can survive upon the exposure of gastric acid and pass into the intestines. The cpe gene is located either in plasmids or in the chromosome and CPE expression may only happen during sporulation. After colonization of *C. perfringens* in the intestine, vegetative cells commit to sporulation and thus to the production of CPE. When sporulation is completed, the mother cells are lysed, and CPE is released into the intestinal lumen. Thereafter, CPE binds strongly to its cellular receptors that are several members of the claudin family. Claudins have been identified as the CPE-binding host proteins that play a critical role in maintaining the normal barrier and forming the backbone of tight junctions in the cell (28). Especially, claudins 3, 4, 6, 8 and 14 are identified as CPE receptors among which claudins 3 and 4 exhibit the strongest binding. Additionally, claudins are composed of four transmembrane domains, a short C-terminal tail and two extracellular loops termed as ECL-1 and ECL-2. Both ECL-1 and ECL-2 regions were found to be necessary for CPE binding. Once CPE binds to claudin receptors, a "small complex" (90 kDa) is composed that is unable to trigger cytotoxicity. However, when several small CPE complexes are oligomerized, a prepore is formed on the plasma membrane surface that results in the construction of a "large complex" (450 kDa) also named as CH-1 (29). At this CH-1 structure, β-hairpin loops assemble into a β-barrel structure that can quickly insert into membranes and thus form a cation-permeating pore (30). Consequently, this CPE pore allows the influx of calcium that activates calpains, leading either to caspase-3 activation and apoptosis in low CPE doses or to necrosis at high CPE doses and excessive calcium influx (Figure 1C).

2.3. *S. aureus* and Staphylococcal Enterotoxins (SEs)

2.3.1. Epidemiology of *S. aureus* and SEs in Foodborne Poisoning Associated Diarrhea

S. aureus is a Gram-positive, cocci-shaped bacterium that tends to form clusters usually described as "grape-like". It is a facultative, coagulase positive microorganism that can grow between 18 and 40 ◦C and can ferment mannitol. In humans, it usually resides on the skin and on mucous membranes, especially in the anterior nares. It is estimated that *S. aureus*-related infections are one of the most common infections in humans, including gastroenteritis, skin and soft tissue infections, pulmonary infections, toxic shock syndrome, urinary tract infections, meningitis. According to the different strains of S. aureus and to the site of infection, *S. aureus* can cause intrusive infections and toxin-regulated diseases. The SEs are potent gastrointestinal toxins and constitute one of the most threatening virulence factors of *S. aureus* for human health (31).

SEs belong to a family of more than 20 different staphylococcal and streptococcal exotoxins with similar functionalities and sequence homology, mostly produced by *S. aureus*. SEs are characterized as pyrogenic enterotoxins and are related to severe human diseases, including food poisoning and toxic shock syndrome. Symptoms of infections related to SEs include diarrhea, vomiting, abdominal pain, cramps and nausea (32). Centers for Disease Control estimated that SEs were associated with 80 million cases in USA, resulting in 325,000 hospitalizations and 5000 deaths (33). Among foodborne diseases, staphylococcal related ones are the second most prevalent. The main cause of this high incidence is attributed either to the insufficient pasteurization of the original product source or due to the contamination that may occur during preparation and handling by individual carriers of the pathogen (34).

2.3.2. Structure of SEs

SEs are globular single-chain proteins broadly classified as SAgs. To date, at least 20 distinct staphylococcal SAgs have been identified, including SEs A through V and toxic shock syndrome toxin-1 (TSST-1) (32). Regarding each enterotoxin subtype, the molecular weight (MW) of the toxins ranges from 20 to 30 kDa with an estimated mature protein length of 220–240 amino acids. It has been estimated that besides the variability of SAgs' primary amino acid sequence, they share similar three-dimensional structures (32). X-ray crystallography results revealed that their quaternary conformation is composed of an α-helical and β-strand structural complex, which creates an ellipsoid protein shape. A common characteristic of those enterotoxins is that they are made up of two unequal domains, stabilized by close packing, in the middle of which a cysteine loop structure is occasionally found, forming a disulfide bridge. When present, the disulfide bond is linked with the amino terminal part of the small protein domain forming an intervening variable loop. On the other hand, the large C-terminal domain is a β-grasp motif of a four- to fivestrand β-sheet that packs against a conserved α-helix (35). For antigen recognition, SAgs interact with major histocompatibility complex (MHC) class II molecules and with T-cell receptors (TCR). For the TCR-binding site, the N-terminal extension has a substantial role, while at

least two distinct binding sites have been recognized for MHC II molecules. The first one is a common, overlapping, generic binding site involving the invariant α-chain of MHC II and the second is a high-affinity, zinc-dependent binding site on the polymorphic β-chain (36).

2.3.3. Mechanism of Action of SEs in Gastro-Intestinal Inflammatory Injury

Food poisoning with SEs results in inflammatory changes in the gastrointestinal tract. *In vitro* and *ex vivo* studies proposed that in order to provoke GI inflammatory injury, SEs first cross the intestinal epithelial barrier and in their intact form, can bind to MHC class II molecules and subsequently attract $CD4^+$ T cells. Thereafter, a strong production of pro-inflammatory cytokines and chemokines occurs, including IL-6, IL8 and MCP-1. Secretion of MCP-1 may lead to increased chemotaxis of $CD4^+$ T cells, macrophages and dendritic cells (DC) from gut-associated lymphoid tissue (GALT) to the inflammation site in GI mucosa. These interactions of SEs with MHC class II may result in hyper activation of professional (macrophages, DC) and non-professional (myofibroblasts) antigen -presenting cells and T cells. Thereafter, an excessive proliferation of $CD4^+$ T cells is provoked, along with an outburst of pro-inflammatory cytokine and chemokine secretion leading to superantigens-mediated acute inflammation and shock (Figure 1D) (32)

2.4. *C. difficile* and Related Enterotoxins (TcdA, TcdB and CDT)

C. difficile, a Gram-positive bacillus, is an anaerobic, spore-forming intestinal colonizer. Studies have shown that *C. difficile* is the primary global cause of nosocomial antibiotic-associated diarrhea and pseudomembranous colitis (37). From spore ingestion to symptoms' occurrence, the time can vary, but typically is short (around 4 weeks). C. difficile is characterized by genetic diversity including both non-pathogenic and pathogenic strains. For the development of a clinical infection, successful germination of *C. difficile* spores resulting in toxin production is needed (38). Symptoms of diseases related to *C. difficile* infections (CDI) may range from mild diarrhea to life-threatening complications such as intestinal perforation, toxic megacolon and pseudomembranous colitis (38). To date, 7 to 10,000 hospitalized patients have been reported with CDI in Europe (39) while in USA, 14,000 deaths are estimated each year caused by hospital-associated CDI (40).

2.4.1. *C. difficile* Toxin A (TcdA) and Toxin B (TcdB)

Epidemiology of TcdA and TcdB

C. difficile toxin A (TcdA) and C. difficile toxin B (TcdB), produced by the pathogenic strains of *C. difficile*, are both efficiently expressed during exponential and stationary phases of bacterial growth (41). Acting as glucosyltransferases, TcdA and TcdB have been identified as the major virulence factors of *C. difficile* infections and are among the largest bacterial toxins rep(41) since now.

Structure of TcdA and TcdB

TcdA and TcdB, two of the largest clostridium enterotoxins, share 48% of their amino acid sequence containing 2710 and 2366 amino acids, respectively. Structures of TcdA (308 kDa) and TcdB (270 kDa) are separated into four domains according to their functionalities based on an ABCD model. For both toxins, the biologically active A domain harbors the glucosyltransferase activity of the toxins and it is located at the N-terminal part, also called N-terminal glucosyltransferase domain (GTD). After the A domain, the C (cutting) domain follows that possesses the protease function. Finally, the D domain arranges the delivery of the toxin or part of it inside the cytosol of the host cell, while at the C-terminal part, the B domain is located and involved in receptor binding. Even if the full-length toxin has not been crystallized yet, single-particle analysis of domains A, C and D allow a nearly complete view of these toxins (42).

Mechanism of Action of TcdA and TcdB

As noted previously, TcdA and TcdB are structured following a multi-domain ABCD model with the goal to inactivate GTPases. Their binding domain is characterized by repetitive sequences, called combined repetitive peptides (CROP), and is located at their C-terminal end, being responsible for toxin attachment on the host cell membrane with different receptors. For TcdA, sucrose-isomaltase (SI) and glycoprotein 96, have been identified as plasma membrane receptors both expressed on human coloncytes (43,44). Two other receptors, chondroitin sulfate proteoglycan 4 (CSPG4) and poliovirus receptorlike protein (PVRL3), have been identified as colonic epithelial receptors for TcdB (45,46). Additionally, for both toxins glycan binding sites have been identified, indicating that more than one receptor is used for cell binding and entry (42). After attachment to their receptors, TcdA and TcdB are internalized into host cells to disturb their function. Despite their homology, both toxins follow different entry mechanisms often directed by the cell surface receptors (37). TcdA uses a clathrin-independent route for entry, controlled by PAC-SIN2 (47), while TcdB uses a clathrin-mediated endocytic pathway (48). Once entering the cell, the toxins reach the acidified endosomes, where they undergo a pH induced conformational change between the CROP and cysteine protease domain region. Structural alterations of toxins lead to pore formation that enables the N-terminal GTD domain to penetrate the endosomal membrane and be released into the host cell cytosol upon translocation (49). An autocatalytic cleavage event was found to be induced by inositol hexakisphosphate (InsP6), a highly charged molecule abundantly found within mammalian cells (50). At the end of their autocatalytic process, TcdA and TcdB glucosylate several members of the Rho subfamily (Figure 2A). The glucosylation of Rho proteins inhibits various cellular functions (e.g., epithelial barrier function, transcription) and signal pathways (e.g., cytokine production), processes crucial for the host (42).

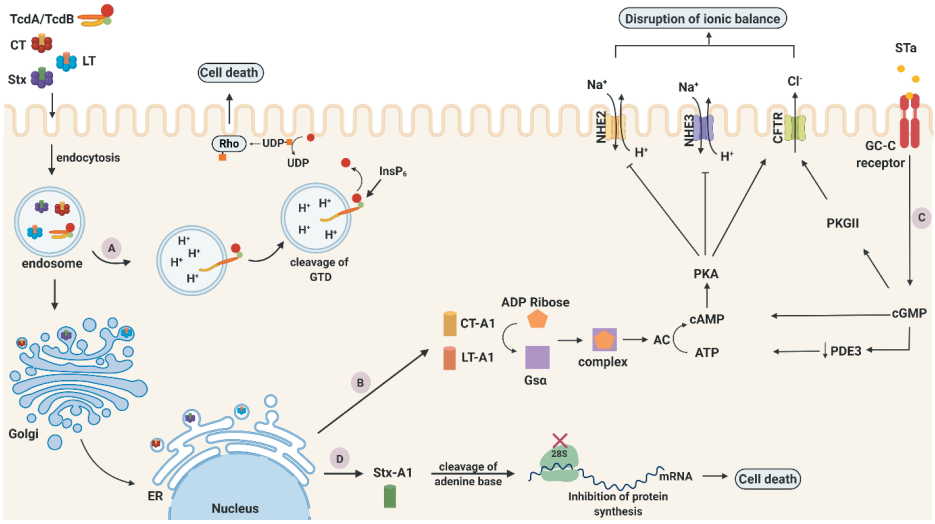


Figure 2. Intoxication pathways of cholera toxin (CT), heat-labile toxin (LT), Shiga toxin (Stx) and *C. difficile* toxins A and B (TcdA/TcdB). (A). TcdA/TcdB cytotoxicity pathways. After internalization through endocytosis, toxins reach acidified endosomes. Low pH induces structural conformations in the toxin delivery domain leading to pore formation and translocation of glucosyltransferase domain (GTD) into the host cytosol. Rho family proteins become inactivated when GTD transfers a glucose unit from uridine diphosphate (UDP)-glucose to the switch I region of GTPase, leading to pathogenic effects or cell death. (B) CT and LT cytotoxicity pathways. After endocytosis and travelling of CT and LT as holotoxins through the trans Golgi network (TGN) and the ER, their catalytic A1 subunit is cleaved and released inside the cytoplasm. Thereafter, the A1 fragment ADP-ribosylate the Gsα subunit of G-protein and consequently activates adenylyl cyclase (AC). Activation of AC leads to elevated levels of cyclic AMP (cAMP) that activate protein kinase A (PKA). PKA stimulates the secretion of Cl^- through cystic fibrosis transmembrane regulator (CFTR) but provokes the inhibition of Na^+ absorption leading to the disturbance of cellular ionic balance, and ultimately apoptosis. (C) STa cytotoxicity pathway. STa binds to guanylate cyclase C (GC-C) receptor and activates its intracellular catalytic domain, which induces cyclic GMP (cGMP). Elevated levels of cGMP activate cAMP-dependent PKA and inhibit phosphodiesterase 3 (PDE3). Subsequently, activated cAMP-dependent PKA along with PKGII, phosphorylate and open cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. Through CFTR, Cl^- and $HCO3^-$ are released in the intestinal lumen, while Na^+ reabsorption is inhibited as PKA has the ability to block the NHE3 channel. These modulating effects on the ionic channels induced by STa, finally result in an electrolyte imbalance that causes cell death. (D) Stx cytotoxicity pathway. Stx is internalized inside host cells (endocytosis) within early endosomes. Afterwards, Stx is following a retrograde transportation pathway, which is directed towards the transGolgi network (TGN) and subsequently reaches the endoplasmic reticulum (ER). In the ER, the enzymatically active A1 fragment translocates into the cytoplasm. Thereafter, it cleaves one adenine residue from the 28S RNA of the ribosomal subunit and thus inhibits protein synthesis leading to cell death. Created with biorender.com (accessed on 20 November 2020).

2.4.2. C. difficile Transferase (CDT)

Epidemiology of CDT

C. difficile transferase (CDT), the third *C. difficile* toxin, is secreted by a number of hypervirulent strains of *C. difficile* such as PCR-ribotypes 078 and 027 responsible for severe CDI outbreaks. Especially over the last decade, the frequency of patients infected by CDT-expressing strains has become increasingly prevalent in the human populations (51).

Structure of CDT

CDT belongs to the family of binary ADP-ribosylating toxins and is composed of two domains, the mature enzyme component CDTa (48 kDa) and the binding component CDTb (98.8 kDa). The N-terminal part of the CDTa contributes to the interaction with the CDTb component, while the C-terminal part is involved in the enzymatic activity. Concerning the CDTb binding compartment, is separated into four domains according to their functionalities. Domain I (residues 0–257) constitutes the activation domain, domain II (residues 258–480) is responsible for membrane insertion and pore formation, domain III (residues 481–591) participates in the oligomerization of the toxin and the C-terminal domain IV (residues 592–876) mediates the binding with the receptor CDTb is activated by proteolytic cleavage of the N-terminal of domain I that allows oligomerization and formation of heptamers (42).

Mechanism of Action of CDT

CDT follows a similar cytotoxicity pathway as the rest of the binary enterotoxins. The attachment of the CDTb domain to the lipolysis-stimulated lipoprotein receptor (LSR) is the first step of its journey inside the host cell (52). LSR is highly expressed in multiple tissues, including colon and small intestine (53). CDTb can bind to LSR in two distinct forms. Either as a monomer, which is proteolytically processed and oligomerized after LSR attachment, or as an heptamer whose precursor form was proteolytically activated and oligomerized before the attachment with LSR. Based on these findings, proteolytic activation of CDTb is not required for receptor binding, although it is essential for oligomerization and subsequent intoxication of host cells. Afterwards, the oligomer-receptor complex and more specifically the N-terminus of activated CDTb, acts as a docking platform for the enzymatic active CDTa component. Once bound to CDTb, the holotoxin is internalized and transported to acidified endosomes inside cells. The acidic environment inside endosomes triggers conformational changes in heptamers leading to a transmembrane pore formation through which CDTa can be translocated across the membrane, and finally reach the host cytosol (37). Productive translocation of CDTa mostly relies on host proteins, including heat shock proteins 70 and 90 (Hsp70 and 90) and cyclophilin A (CypA) (54). Inside the cytosol, CDTa, as an ADP-ribosyltransferase, catalyzes the transfer of ADP-ribose from nicotinamide adenine dinuclotide (NAD^+) to Arg-177 of G-actin monomer inhibiting its polymerization. Intoxication of CDT may lead to loss of actin-based cytoskeleton and high toxin concentrations can even lead to cell death. At low concentrations, rearrangement of actin cytoskeleton

triggers the formation of microtubule-based cell protrusions, which form a network on the surface of epithelial cells that enhances pathogen adherence and colonization (55).

2.5. *V. cholerae* and Cholera Toxin (CT)

2.5.1. Epidemiology of *V. cholerae* and CT

V. cholerae is a Gram-negative bacterium that belongs to the Vibrionaceae family and more specifically to the gamma subdivision of the phylum Proteobacteria. It is a highly motile, curved oxidase-positive, facultative anaerobe that ferments glucose, sucrose and mannitol. *V. cholerae* is the causative agent of cholera but only the strains that produce cholera toxin are associated with the disease. Infection with *V. cholerae* is caused by the ingestion of contaminated food and water, especially in areas with poor sanitation and hygiene (56).

To date, seven cholera pandemics have been officially recognized (57). After being digested and survived in the acidic gastric environment, *V. cholerae* colonizes in the small intestine epithelium producing CT. Secretion of CT leads to an extensive discharge of watery stool with direct consequences of electrolytes' loss, rapid dehydration, fall of blood pressure, vomiting and cramps in legs and abdomen (58) In severe cases, rapid loss of fluid leads to hypovolemic shock and metabolic acidosis (59). World Health Organization estimates 1.4 to 4.4 million cholera cases with death rates between 21,000 and 143,000 per year (60).

2.5.2. Structure of CT

CT belongs to the family of AB5 toxins and is an 84-kDa heterogeneous protein made up by two subunits. The first subunit is the CTA active domain (28 kDa) with a length of 240 amino acids, while the second one is the CTB homopentameric non-toxic B domain (56 kDa) of 103 amino acids in length per each monomer. Initially, CTA is synthetized as a single polypeptide chain, however after post-translational modification, CTA1 (residues 1–192; 23 kDa) and CTA2 (residues 193–240; 5 kDa) fragments are generated and remain linked together with a disulfide bond (61). The toxic activity of CT resides in CTA1, whereas CTA2 mediates the insertion of CTA into the CTB pentamer. The circular B-subunit homopentamer is responsible for the binding of the toxin to cells and is held together by approximately 130 hydrogen bonds and 20 inter-subunit salt bridges, which provide an outstanding stability to the pentamer. The CTB pentamer and the CTA2 fragment are non-covalently linked except the sequence of the last four amino acids (lysine-aspartateglutamate-leucine) at the carboxy-terminal of CTA2 that pro-trudes from the associated toxin and is not engaged in interactions with the pentamer (62).

2.5.3. Trafficking and Mechanism of Action of CT

The pathogenic journey of CT inside epithelial cells, after *V. cholerae* colonization, begins with the attachment of the toxin to the GM1 ganglioside: β-D-Gal-(1→3)-β-DGalNAc-(1→4)-[α-D-Neu5Ac-(2→3)]-β-D-Gal-(1→4)-β-D-Glc-ceramide. GM1 has been identified as the receptor of CT that facilitates its internalization inside the host cell membrane of epithelial

cells (63). For each B monomer there is one binding pocket for the GM1 receptor resulting in a total of five binding sites. The attachment of the toxin with the receptor mostly involves Gal and sialic acid, the two terminal sugars of GM1 (64). After being attached, the GM1-toxin complex internalizes inside the cell from the plasma membrane to early endosomes following different entry routes (65). Previous studies on distinct types of cells have reported various uptake mechanisms, such as, caveolae-dependent (66), clathrin-dependent (67) , or non-caveolae/non clathrin-mediated pathways (68). Regardless of the mode of entry, the toxin internalizes in early endosomes and travels to the transGolgi network following a retrograde transportation pathway (69). The appearance of the last four amino acids residues (Lys-Asp-Glu-Leu) at the C-terminal of the A2 chain, termed as KDEL in the one-letter code, has been reported as an endoplasmic reticulum (ER) targeting motif, and its presence indicates the trafficking of the holotoxin to the ER (70). By entering the lumen of ER as a holotoxin, the A subunit is proteolytically cleaved between residues Arg192 and Ser193 by a bacterial endoprotease to the wedge-shaped A1 subunit and to the elongated A2 subunit. The two subunits remain linked with an intrachain disulfide bond (Cys187 = Cys199) until the trafficking of the A1 subunit inside the cytosol, where the disulfide bond is reduced by the purified protein disulfide isomerase (69). Through SEC61 channel, the A1 chain reaches the cytosol where it catalyzes ADP-ribosylation of the a-subunit of the stimulatory G protein (Gs). This post translational modification occurs by transferring an ADP-ribose from nicotinamide adenine dinuclotide (NAD^+) to an arginine residue of the protein. Being ribosylated, the stimulatory G protein in its guanosine triphosphate (GTP)-bound form, activates adenylyl cyclase (AC), which subsequently induces the conversion of ATP into cyclic AMP (cAMP). Elevated levels of intracellular cAMP in crypt cells activate protein kinase A (PKA) that phosphorylates the cAMP-dependent chloride channel (CFTR). Through cystic fibrosis transmembrane conductance regulator (CFTR), an enhanced efflux of electrolytes and water occurs in the intestinal lumen with a direct result of the production of a large volume of watery diarrhea (Figure 2B) (61).

2.6. *E. coli* and Related Enterotoxins

E. coli, a Gram-negative bacterium, is an aerobic, non-spore-forming bacillus that belongs to the family of Enterobacteriaceae. *E. coli* strains are classified into different categories according to the unique interactions with eukaryotic cells. Some of the most known *E. coli*-related enterotoxins are the enterotoxigenic *E. coli* (ETEC), the enteropathogenic *E. coli* (EPEC), the enterohemorrhagic *E. coli* (EHEC), the Shiga toxin-producing *E. coli* (STEC) and the uropathogenic *E. coli* (UPEC) (56). Based on its pathogenic potential, *E. coli* is divided into non-pathogenic groups in which it acts as a commensal member of the gut microbiota and to pathogenic groups. *E. coli* is associated with a wide range of infections including intestinal pathologies (e.g., gastroenteritis) and extra-intestinal pathologies (e.g., urinary tract infections, neonatal meningitis and bacteremia). Even if some of those infections can be very common like urinary tract infections, some others like hemolytic uremic syndrome (HUS) can be associated with high morbidity and mortality (71).

2.6.1. *E. coli*, LTs and STs

Epidemiology LTs and STs

Enterotoxigenic *Escherichia coli* (ETEC) is the major causative agent of induced diarrhea mainly associated with the production of several enterotoxins including LTs and STs. Approximately 840 million infections of ETEC-induced diarrhea are estimated each year as well as around 3,800,000 deaths worldwide (72).

LTs produced by *V. cholerae* and *E. coli* are members of a family of structurally identical proteins that provoke diarrheal symptoms in humans. The family is composed of two major subcategories according to their biochemical, genetic and immunological characteristics. The Type I subcategory is comprised of CT from *V. cholerae* and LT-I enterotoxin (or LT) from *E. coli*. Type II consists of LT-IIa enterotoxin and its partially cross-reacting antigenic variant, LT-IIb, both produced from *E. coli*. The A and B polypeptides of both CT and all LTs are synthesized inside the cytoplasm as precursors, including an N-terminal signal sequence. From the cytoplasm, they get secreted into the periplasmic space where they assemble spontaneously to the holotoxin molecules by removing the signal sequence from their structure (73). In addition to *V. cholerae* and *E. coli*, other enteric pathogens like *Aeromonas hydrophilia* and *Plesiomonas shigelloides* have also been found to produce cholera toxin-like LTs. Along with structural similarities of LT-I and LT-II with CT, LTs also follow identical intoxication pathways inside host cells. However, minor differences in terms of receptor type, C-terminal motifs and ADP-ribose acceptors, distinguish toxins' pathogenicity routes (73).

Strains of ETEC-producing STs are ranked among the top eight enteropathogens that cause diarrhea, accounting for a mortality rate of 3.2% in 2016. Especially in children, ETEC infection may lead to long term consequences including stunted growth, chronic gut inflammation and impaired cognate development (74). Except for their distinct role in diseases, heat-stable enterotoxin type a (STa) is characterized as methanol soluble and protease resistant, while heat-stable enterotoxin type b (STb) is exactly the opposite. This review focuses mostly on STa as our review concerns human-related diseases.

Structure of LTs and STs

LTs belong to the family of AB5 toxins and are mostly related to CT both in terms of structure and of function. Therefore, LTs are oligomeric toxins (86 kDa) composed of two main subunits, the A subunit (28 kDa) and the pentameric B subunit (58 kDa). Enzymatic catalytic activity of LTs is attributed to free LTA, while LTB pentamer is responsible for the entry of the toxin into host cells Mudrak(75). LTs, like CT, bind to the GM1 receptor with high affinity, with five binding sites per each B pentamer. Except GM1, LTs can alternatively bind to blood group A and B determinants through a novel binding site which is located in a distinct position from the GM1 binding site (72).

STs constitute a family of low-molecular-mass peptide toxins, divided into two categories, Sta and STb. Sta is more associated with diarrhea induced in humans and is subcategorized into two variants, STh, a 19 amino-acid protein (2 kDa), found in human ETEC strains and STp, an 18 amino-acid protein (2 kDa), isolated from human and porcine strains (76). STh and STp are almost identical since they include 14 similar amino acid residues. In addition, all of them contain a cysteine-rich core that is crucial for the expression of their biologic activity. Inside the core, cysteine residues are all arranged in disulfide bridges whose integrity is an essential requirement for the maintenance of toxin conformation (77). On the other hand, STb is a 48 amino-acid protein (5.1 kDa) which is virulent only against animals (76).

Trafficking and Mechanism of Action LTs

To begin cell intoxication, the binding domain of LTs binds to the glycolipid receptors in plasma membranes and the toxin-receptor complex enters inside the cell through endocytosis. Following a retrograde transportation pathway, the holotoxin travels to the Golgi apparatus and subsequently to the ER. Different C-terminals motifs of the A2 tail are recognized by a luminal ER membrane protein (ERD2) that can retrieve toxins from the Golgi apparatus. The four last amino acid residues (Lys-Asp-Glu-Leu) of CT and LT-IIb are termed KDEL in the one-letter code while for LT-I and LT-IIa are termed RDEL (Arg-AspGlu-Leu). After proteolytic cleavage of A subunit, reduced A1 crosses the ER membrane lipid bilayers and gains access inside the cytosol by exploiting the Sec61 machinery. Despite the fact that normally proteins that are exported from the ER by similar processes are targeted for degradation by the proteasome, the scarcity of lysines in the active part of toxin, prevents toxins from ubiquitination and degradation. Inside the cytosol, the expression of A1 toxic effects begins with the ADP-ribosylation of an arginine residue in the stimulatory alpha (Gsα) subunit of the heterotrimeric regulatory G protein complex. A1 subunits may use different substrates as ADP-ribose acceptors including arginine and other guanidine compounds, such as, agmatine. The most substantial difference among LTs members is the much lower activity of the LT-II toxins that use agmatine as acceptor. Inactivation of intrinsic GTPase activity of Gsα stimulates AC that subsequently leads to elevated levels of cAMP. As a consequence, accumulation of cAMP, activates PKA-dependent pathways that stimulates Cl^- secretion by phosphorylation of the CFTR channel resulting in the loss of fluid and electrolytes inside the intestinal lumen (Figure 2B). Disturbance of electrolyte and water balance inside the cell provokes severe volumes of watery diarrhea (73).

Trafficking and Mechanism of Action of STs

Intoxication pathway of STa begins with toxin binding to the guanylate cyclase C (GC-C) receptor in the membrane of the small intestinal epithelial cells (IECs). Through the activation of the intracellular catalytic domain, Sta causes the hydrolysis of GTP that induces the accumulation of intracellular cyclic GMP (cGMP) levels. Increased levels of cGMP activate cGMP-dependent protein kinase II (PKGII) and inhibit phosphodiesterase 3 (PDE3) which in turn activates cAMP-dependent PKA. In their activated forms, PKGII and PKA phosphorylate and open the CFTR Cl^- channel through which, Cl^- and $HCO3^-$ are released

into the intestinal lumen. Finally, PKA can inhibit Na^+ reabsorption by phosphorylating Na^+/H^+ exchanger 3 (NHE3) (74). The inhibitory and activation effects of STa on the ionic channels finally leads to electrolyte imbalance and cell apoptosis (Figure 2C).

2.6.2. *E. coli* and Stxs

Epidemiology of Stxs

The prototypical member of Stx family was first released from Shigella dysenteriae serotype1 followed by a Shiga-like toxin type, almost identical to the original one, produced by Stx-producing *E. coli* (STEC) (78). Stx-producing bacteria enter the intestine through ingestion of contaminated food and water, which may lead to various diseases ranging from asymptomatic carriage to hemorrhagic colitis and HUS (79). Annually, STEC causes 2,801,000 acute illnesses worldwide that lead to 3890 cases of HUS (80). Even if the majority of patients can recuperate from uncomplicated diarrheal disease within a week, 6 to 9% of the patients develop HUS with devastating consequences, such as, thrombocytopenia, microangiopathic hemolytic anemia, and acute renal injury. Especially in children, STEC infection is the primary cause of acute kidney failure along with the high incidence of such infections in the elderly population (79).

Stx1 and Stx2 are the two main types of Stx family released from Stx-producing bacteria. Each type of toxin is separated to several subtypes differentiated according to their amino acid diversity. Stx1a, Stx1c and Stx1d are attributed to Stx1 whereas Stx2aStx2g to Stx2 (81). Genes coding for Stx (stx gene) are embedded within the genomes of lambdoid bacteriophages instead of the actual bacterial genome, and remain silent during the lysogenic cycle but Stx production is initiated after lytic cycle induction (82).

Structure of Stxs

The structure of Stxs corresponds to an AB5 protein model. The A subunit (~32 kDa), non-covalently linked to the homopentameric B subunit (~7.7 kDa), is responsible for the enzymatic activity of the toxin. In order to be enzymatically active, the A subunit is composed of two separate fragments, the A1 (27.5 kDa) and the A2 (4.5 kDa) fragments that remain linked with a disulfide bond (Cys242 = Cys261). The A1 fragment possesses the Nglycosidase activity of Stxs, while the C-terminus of the A2 fragment mediates the binding with the B compartment. On the other hand, the B subunit binds to the glycosphingolipid Gb3 (α-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-Glc-ceramide) receptor that is identified as the native receptor of Stxs that facilitates toxin host internalization. For each B monomer, three binding sites have been identified resulting in 15 total binding sites for every toxin unity (83).

Trafficking and Mechanism of Action of Stxs

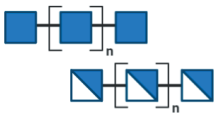
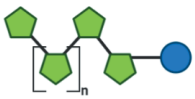
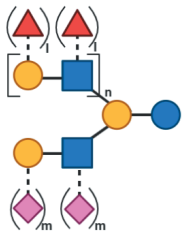
Stxs are released into the intestinal lumen following different endocytic pathways according to the presence or absence of glycosphingolipid Gb3. Gb3 acting as Stx receptor is located on the host cell membranes. In tumorigenic colon cancer cells where Gb3 is

expressed, Stx exploits the binding with the Gb3 receptor to internalize inside host cells. At human IECs where Gb3 is not expressed, Stx follows Gb3-independent pathways, such as, macropinocytosis. Generally, IECs utilize macropinocytosis, a clathrin-independent mechanism, for the internalization of large molecules (84). Previous studies have proven the association of Stxs with actin-coated macropinosomes in the intestinal epithelium and their subsequent transportation from apical to basolateral surfaces (85). Regardless of the endocytic pathway, the toxin is transferred to the trans-Golgi network followed by the ER lumen retrograde intracellular transportation (86). During trafficking to the ER, furin cleaves the A subunit into A1 and A2 fragments which remain linked via a disulfide bond that is reduced once the toxin enters the ER (79). Through the ER-associated protein degradation pathway, unfolded A1 toxin exits the ER and enters the cytoplasm where it cleaves an adenine base of 28S ribosomal RNA of eukaryotic ribosomes. As a result, protein chain synthesis in the cell is inhibited, since the injured ribosome is no longer associated with the elongation factor-dependent amino-acyl tRNA (81). The presence of the A1 polypeptide inside the cytoplasm not only inhibits the protein synthesis, but also activates several stress response pathways, such as ribotoxic stress and the ER stress response (87) as depicted in Figure 2D. After entering the bloodstream, toxins aim to reach their targets, including the kidneys and the brain, leading to systemic complications and in severe cases to death.

3. Non-Digestible Oligosaccharides

NDOs are carbohydrate moieties composed of less than 20 monosaccharide building blocks linked via glycosidic bonds (88). The number of monomeric sugars of every NDO structure determines the DP of each moiety, which subsequently may influence their antivirulent behavior (89). An overview of the basic structures of the NDOs that demonstrated a certain role in fighting against EPB and associated enterotoxins is depicted in Table 1. NDOs that exhibit anti-pathogenic/anti-virulence effects are alginate-oligosaccharides (AOS), chito-oligosaccharides (COS), fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), human milk oligosaccharides (HMOs), isomalto-oligosaccharides (IMOS), mannan-oligosaccharides (MOS), pectic-oligosaccharides (POS) and xylo-oligosaccharides (XOS).

Table 1. Structural overview of different non-digestible oligosaccharides (NDOs).

Non-Digestible Oligosaccharides	Structures
Alginate-oligosaccharides (AOS)	
Chito-oligosaccharides/Chitosan-oligosaccharides (COS)	
Fructo-oligosaccharides (FOS)	
Galacto-oligosaccharides (GOS)	
Human milk oligosaccharides (HMOs)	
Isomalto-oligosaccharides (IMOS)	
Mannan-oligosaccharides (MOS)	
Pectic-oligosaccharides (POS)	
Xylo-oligosaccharides (XOS)	

AOS are derived from the enzymatic depolymerization or the acid hydrolysis of alginate, a biopolymer present in the cell walls of brown algae [90]. Alginate contains two monosaccharide building blocks, (1→4)–linked β-D-Mannuronic acid (ManA/M) and (1→4) α-L-Guluronic acid (GulA/G). These can be either homogenously or heterogeneously linked forming homodimers (GG/MM) or heterodimers (MG/GM) (90).

COS are degraded products of chitin and of chitosan, produced after enzymatic and chemical hydrolysis (91). Chitin is present on crustacean or arthropodic shells and contains a high proportion of β-(1→4)-linked N-acetylglucosamine (GlcNAc), while chitosan is present in

cell walls of specific fungi and is mainly composed of the β-(1→4)–linked Dglucosamine (GlcN) (92). The average molecular weight (MW) of COS is less than 3900 Da, while the DP is less than 20 (91).

FOS, also known as oligofructose or oligo fructan, are naturally found in higher plants, like fruits and vegetables and can be either obtained by plant extraction or by enzymatic manufacture (93) The chemical structure of FOS is composed of a linear chain of fructose units linked by β-(2→1) glycosidic bonds, terminated by a glucose (Glc) unit linked with an α-(1→2) glycosidic bond. Structures of FOS with a DP of more than 10 are termed as inulin (94).

GOS can be synthesized from lactose by a β-galactosidase enzyme in a reaction known as transgalactosylation (95). Monosaccharide building blocks included in the structure of GOS are galactose (Gal) units (DP = 2–6), linked by different bonds such as β-(1→3), β-(1→4) and β-(1→6) glycosidic linkages terminate in a β-(1→4) linked glucose unit (96). Since FOS and GOS resemble the oligosaccharides that are naturally present in human breast milk, several types of infant formula are supplemented with those two oligosaccharides to obtain the advantages of a breast-fed microflora (97).

HMOs constitute a key component of human milk and represent a group of structural and biological diverse and complex indigestible sugars. According to the different monosaccharide moieties of their structure, HMOs can generally be divided into neutral oligosaccharides, containing occasionally fucose (Fuc) units (fucosylated HMOs) and acidic oligosaccharides (sialylated HMOs), containing sialic acid units. The basic monosaccharide components of HMOs are Gal, Glc, Fuc and GlcNAc, or sialic acid (98).

IMOS are naturally present at low concentrations in honey and in fermented foods like in soy sauce. Alternatively, IMOS can be manufactured by an enzymatic process utilizing starch as the substrate. The main monosaccharides components of IMOS are Glc units linked with α-D-(1→6) glycosidic bonds, with a DP range from 3 to 6 (93). MOS can be chemically synthesized or obtained from the outer cell-wall membrane of bacteria, plants or yeast (99). Isolated structures of MOS are mostly composed of (1→2), (1→4) and (1→6) D-mannose linkages (100).

POS are obtained by the depolymerization of pectin, a plant complex macromolecule made up of several monosaccharides (101). Pectins are most importantly composed of a linear backbone of α-(1→4)-linked D-Galacturonic acid (GalA) units that can be partially acetylated and/or methylated (102). The linear structures of pectins, termed as “smooth” homogalacturonic regions are made up by GalA and are occasionally interrupted by rhamnose residues called “hairy” rhamnogalacturonic regions (102).

XOS are naturally found in bamboo shoots, fruits, vegetables, milk and honey and are formed by 2 to 10 xylose molecules, linked by β-(1→4) glyocosidic bonds (103). Additionally,

XOS can be produced chemically by direct enzymatic hydrolysis of xylan (104). Structural characteristics of the mentioned NDOs play a crucial role in their mechanism of action against EPB and enterotoxins, as will be discussed in the next paragraph

4. Different Effects of NDOs and SCFA against Enterotoxins and Enterotoxin-Producing Bacteria

4.1. The Effects of NDOs and SCFA against Enterotoxin-Producing Bacteria

NDOs are proven to have crucial roles in protecting the body from pathogenic bacteria. They play this role via at least two different pathways inducing both direct and indirect defenses against these pathogens. First, NDOs can also encounter toxigenic attacks through their direct interaction with EPB inducing anti-adhesive, anti-biofilm and anti-growth effects. Their anti-adhesive capability is based on the similarity of their carbohydrate backbone with the structure of EPB receptors on host cells. Furthermore, NDOs can disrupt or inhibit the formation of biofilms, an extracellular polymeric sub-stance (EPS) matrix that pathogenic bacteria develop as a protective mechanism (105). Interestingly, NDOs can also exert antibacterial effects that directly result in growth inhibition of pathogenic bacteria, including enterotoxin-producing microorganisms (106). Second, NDOs can modulate the microbiota balance by encouraging the growth of beneficial bacteria in the gut lumen. Several studies proved that this modulation will not only result in an increase in SCFA production, but also in a decrease in the essential sources and space that pathogenic bacteria use to proliferate inside the intestinal lumen. This indirect effect of NDOs on the proliferation of EPB was investigated in several studies, which are depicted in Table 2. Concerning the indirect mode of action, oligosaccharide substrates get fermented by the potential beneficial bacteria species, resulting in an increased production of SCFA (2). SCFA are volatile fatty acids produced in the large bowel, structurally characterized by fewer than six carbons, existing both in straight and branched-chain versions. After being produced, SCFA are absorbed and used in various biosynthetic pathways by the host, constituting an energy source (107). Oligosaccharide-induced microbiome composition can benefit host health and protect against EPB by microbiota-dependent mechanisms.

Table 2. Indirect effects of NDOs on enterotoxin-producing bacteria

NDOs	Beneficial Bacteria	Bacteria Producing Enterotoxins	Mechanism of Action	Health Benefit	Ref.
FOS	1. *Lactobacillus rhamnosus* 2. *Lactobacillus reuteri* 3. *Lactobacillus rhamnosus* (probiotic mixture) 4. mixture of six *Lactobacilli* and three *Bifidobacteria* strains	Enteropathogenic *Escherichia coli* (EPEC), *Clostridium difficile*	Not investigated	Growth inhibition	(108)
FOS, inulin	*Lactobacillus rhamnosus*	Enterotoxigenic *Escherichia coli* (ETEC)	Decrease in cAMP and cGMP levels	Anti-virulence activity	(109)
FOS, inulin	1. *Lactobcillus acidophilus*, 2.*Lactobacillus lactis*, 3. *Lactobacillus casei*, 4. *Lactobacillus reuteri*	*Staphylococcus aureus*, *Escherichia coli*	Production of metabolites by beneficial bacteria	Anti-biofilm, anti-adhesive, antimicrobial effect	(110)
FOS, XOS, inulin, mixtures of inulin:FOS (80:20 w/w) and FOS:XOS (50:50 w/w)	1. *Lactobacillus plantarum*, 2. *Bifidobacterium bifidum*	*Escherichia coli*	Production of SCFA	Growth inhibition of Escherichia coli	(111)
XOS	1. *Lactobacillus* spp. 2. *Bifidobacterium*	spp. *Clostridium perfringens*	Production of SCFA	Growth suppression of Clostridium Perfringens	(103)
GOS	*Bifidobacterium breve*	Methicillin resistant *Staphylococcus aureus* (MRSA)	High production of acetic acid (low pH)	Prevention of systemic MRSA infection	(112)
GOS, IMOS	1. *Lactobacillus plantarum*, 2. *Lactobacillus paracasei*, 3. *Bifidobacterium breve*, 4. *Bifidobacterium lactis*	*Clostridium difficile*	Stimulation of gut microflora	Protective effect against CDI	(113)

4.1.1. Direct Mechanisms of Action of NDOs against EPB

Various bacteria found in the intestinal tract are able to interact with the digestive mucosa and produce virulence factors responsible for gastrointestinal or foodborne diseases. Among the diverse virulence factors, enterotoxins represent the most invasive way to affect target cells. One category of toxigenic bacteria that produce enterotoxins are colonizing bacteria (e.g., *E. coli*, *V. cholerae*) whose adherent factors (e.g., pili, fimbriae) permit the evasion of inhibitory microflora. The second category are bacteria (e.g., *B. cereus*, *S. aureus*) that can grow and secrete their toxins in different environments, such as, food, leading to the digestion of pre-formed toxins that cause food intoxication. Finally, a third class of toxigenic bacteria (e.g., *C. difficile*, *C. perfringens*) can enter the digestive tract and grow in the intestinal lumen under certain conditions such as antibiotic treatment, to overcome the inhibitory effects of resident microflora (6). Even if enterotoxins constitute one of the major therapeutic targets against EPB, several additional targets (e.g., adherence, biofilm formation) have also been investigated. NDOs have shown promising therapeutic potentials through their interaction with EPB. Direct anti-pathogenic capabilities of NDOs against EPB target both bacterial and enterotoxin toxicity pathways and mostly rely on the structural similarity of free NDOs with the carbohydrate patterns presented on the host cell surface (106) A concise overview of the direct effects of NDOs against EPB is depicted in Table 3.

Table 3. Direct effects of NDOs on enterotoxin-producing bacteria.

Bacteria	Toxins	NDOs	Mechanism of Action	Ref.
Escherichia coli	Stx, LTs, STs	Pooled HMOs, 30SL, 60SL, DSLNT, 6SLN, 30S3FL, LST a	Inhibition of hemagglutination	(114)
		Fucosylated HMOs	Anti-adhesive effect	(115)
		Pooled HMOs, 3-FL, 30SL, 60SL, acidic and neutral (HMW and LMW) HMOs	Anti-adhesive effect	(116)
		2 0FL, 3-FL	Anti-adhesive effect	(117)
		2 0FL, 60SL	Anti-adhesive effect	(118)
		Pooled HMOs	Reduced enteropathogenic E. coli (EPEC) attachment to epithelial cells	(119)
		AOS	Inhibition of biofilm formation	(120)
		COS	Antimicrobial effect	(121)
			Anti-adhesive effect	(122)
			Antibacterial effect (growth inhibition)	(123,124)
			Antioxidant and antimicrobial effect	(125)
			Membrane disruption (growth inhibition)	(126)
		GOS	Anti-adhesive effect	(127)
		MOS	Anti-adhesive effect	(128,129)
			Growth inhibition	(130)
		POS	Anti-adhesive effect	(131)
			Antimicrobial effect	(132,133)
Vibrio cholerae	CT	Pooled HMOs	Anti-adhesive effect (neutral, HMW)	(116)
		Isolated HMOs	No anti-adhesive effect (LMW)	(116)
		COS, NAc-COS	Bactericidal effect	(134)
Bacillus cereus	Hbl, Nhe, CytK	COS	Antibacterial effect (growth inhibition)	(134,135)
			Membrane disruption (growth inhibition)	(126)

Table 3. Continued

Bacteria	Toxins	NDOs	Mechanism of Action	Ref.
Staphylococcus aureus	SEs	Pooled HMOs	Inhibition of biofilm formation	(105)
		COS	Antibacterial effect (growth inhibition)	(123,134, 136)
			Antioxidant and antimicrobial effect	(125)
		POS	Growth inhibition	(130)
			Antimicrobial effect	(132)
			Anti-adhesive effect	(137)
		XOS	Antibacterial effect	(138)
Clostridium spp.	TcdA, TcdB, CDT, CPE, CPB	FOS	Anti-adhesive effect Inhibition of biofilm formation	(139)

E. coli

A great number of NDOs can exhibit antibacterial effects against different pathogenic strains of E. coli.

The anti-adhesive capabilities of NDOs, such as GOS are mostly related to molecular mimicry mechanisms in which free oligosaccharides resemble the structure of host cells carbohydrate based receptors (127). The way that *E. coli* binds to epithelial cells mostly relies on the adhesins found on the fimbriae appendage of its structure. Different fractions of HMOs, including neutral, fucosylated and acidic fractions are known to inhibit adhesion of *E. coli* to epithelial cells. Fractions of neutral HMOs inhibit the adherence of *an E. coli* strain which is P-fimbriated and specifically recognizes galabiose or galactose structures on host cell surface (114). Fucosylated HMOs, a subcategory of neutral HMOs decorated by fucosyl residues, inhibit Enteropathogenic *E. coli* (EPEC) adhesion on HEP-2 monolayers (115). However, the great abundance of different molecular structures incorporated in HMOs mixtures makes it difficult to specify the effectiveness of a specific saccharide. Negatively charged sialylated HMOs can interact with oppositely charged elements on the epithelial cell exterior, showing inhibitory potential towards pathogenic species (116). However, acidic HMOs can partially inhibit P and CFA fimbriae-expressing *E. coli* as the P-fimbrial lectin lacks affinity for sialylated oligosaccharides (114).

Anti-adhesive effects against *E. coli* are also exhibited by POS following a P-fimbriae mediated inhibition mechanism (131). However, although the POS structure lacks the exact α-Gal-(1-4)-β-Gal termini that P-fimbriated E. coli utilizes to adhere to epithelial cells, receptor mimicry is likely involved, but additional mechanisms remain unknown(140). Furthermore, MOS can inhibit E. coli adherence to epithelial cells as the FimH domain of

type I fimbriae, commonly found in *E. coli*, recognizes mannose patterns on host cells in order to be adhered. MOS can bind to the FimH domain and compete with the mannose patterns, inhibiting pathogenic adhesion through a receptor-mimicking function (129). Finally, COS was also found to inhibit EPEC adhesion, while it did not exert the same capability against Verocytotoxin-producing *E. coli* (VTEC), showing target specificity (122). Even if the reason for strain selectivity is currently undetermined, the anti-adhesive effect is presumably again a result of molecular mimicry, while the GlcNAc moiety on the cell surface receptor is recognized by the GafD adhesin of the G fimbriae containing *E. coli* strain and simultaneously constitutes one of the basic compartments of COS structure (122).

In addition to anti-adhesive capabilities of NDOs, other anti-pathogenic effects against *E. coli* have also been identified including bacterial cell membrane disruption, biofilm inhibition and radical scavenging. A great number of studies have shown that COS act as antibacterial agents via the inhibition of *E. coli* growth (123,124) The positively charged amino groups of chito-oligomers can bind to the negatively charged O-specific antigenic units of the *E. coli*, thereby blocking the nutrient flow leading to bacterial death due to nutrient depletion (126). Additionally, AOS block pathogenic swarming and motility in *E. coli*, two substantial mediators for biofilm formulation (120). POS can inhibit *E. coli* growth by scavenging free radicals like HO•, reacting with them and producing carbon dioxide radical anion ($CO2^{\bullet -}$). However, *E. coli* is inhibited less significantly by POS in comparison with *S. aureus* (130).

V. cholerea

NDOs exhibit anti-adhesive and antibacterial effects against pathogenic *V. cholerea*. Among several NDOs, neutral high-molecular weight (HMW) HMOs and COS have shown such anti-pathogenic potential against *V. cholerea*, following receptor mimicking and membrane disruption mechanisms (116,134). In order to achieve colonization in the small intestine, *V. cholerea* expresses the N-acetylglucosamine-binding protein A (GbpA), a nonspecific adhesin that facilitates attachment to the intestinal epithelium by specific binding to GlcNAc oligosaccharides (141). GlcNac consists of a structural component of glycoproteins and glycolipids that are located on the IECs and on mucus. Except the abundance of GlcNaC on cellular moieties, it also constitutes a basic component of COS and HMOs (56). Therefore, NDOs that include GlcNAc in their structure can mimic host cell receptors and compete for their binding with GbpA. Through a receptor-mimicry mechanism, the HMW fraction of HMOs was shown to inhibit the adhesion of *V. cholerae* to Caco-2 cells, thereby reducing its pathogenicity (116).

Furthermore, the antibacterial potentials of COS against pathogenic bacteria were related to the increased solubility and their cationic nature. The polycationic nature of COS enables them to adhere to Gram-negative bacteria, such as *V. cholerae*, creating a cationic oligosaccharide layer around them (142). As it is adhered to the bacterial cell surface after prolonged exposure, COS can promote the leakage of proteinaceous and other intracellular

constituents, resulting in cell swelling, cell lysis and inhibition of bacterial growth (143). Consequently, this mechanism is an estimated explanation for the bactericidal activity of COS and NAc-COS against *V. cholerae*, possibly due to the lower MW and the higher solubility of chito-oligomers in comparison with the polymeric chitosan moieties (134).

B. cereus

Among different antibacterial properties of NDOs, the anti-pathogenic effects of COS prevail against *B. cereus*. COS have shown to inhibit microbial cells either by interfering with the cell surface of bacteria or by blocking the transcription of RNA from DNA (134). The cationic nature of chito-oligomers due to the positively charged $NH3^{+}$ groups, enable them to bind with the peptidoglycan layers of Gram-positive bacteria, such as *B. cereus*, a Gram-positive bacterium. The cell wall of B. cereus is composed of peptidoglycan layers to which polycationic moieties (positively charged $NH3^{+}$ groups) of chito-oligomers can bind. The binding that occurs leads to cell wall disruption, exposure of cell membrane to osmotic shock and secretion of intracellular substances that ultimately result in growth inhibition of Gram-positive bacteria, such as *B. cereus* (126).

S. aureus

Multiple NDOs have shown antibacterial activities against *S. aureus*, a Gram-positive bacterium that is characterized by its biofilm formation and multi-drug resistance (144). In order to encounter *S. aureus* escape mechanisms, the ability of COS to potentiate conventional antibiotics was investigated. Indeed, results have shown that COS enhanced the activity of several antibiotics possibly through the lysis of the bacterial cell wall (145). Generally, anti-pathogenic features of COS are associated with a number of factors, including MW and degree of deacetylation (DD) (146). COS with a MW less than 5000 can penetrate through the bacterial membrane in order to bind to bacterial DNA and inhibit RNA synthesis (147). Bearing a positive charge, COS are also able to create an impermeable cationic oligosaccharide layer around the surface of *S. aureus* bacteria, thereby preventing the diffusion of metal ions and other nutrients, elements that are essential for bacterial proliferation, across the bacterial membrane (136).

Plant-based oligosaccharides, like POS, can also inhibit the growth and the adhesion of *S. aureus*. Characterized by redox activity, POS act as antioxidants, since they can efficiently scavenge free radicals. To eliminate pathological effects induced by free radicals such as HO•, POS react with HO• and produce the carbon dioxide radical anion ($CO2^{\bullet -}$), which is hypothesized to inhibit *S. aureus* (130). However, since POS are characterized by great versatility, the antibacterial potential of $CO2^{\bullet -}$ needs to be further elucidated. Moreover, anti-adhesive effects of POS are mostly attributed to the high uronic acid content of their structure that results in higher ionic interactions among POS and pathogenic bacteria. The anti-adhesive mechanism of POS, derived from panax ginseng, was proposed against pathogenic Gram-positive bacteria, including S. aureus (137). However, similar anti-

adhesive effects were not exerted against beneficial and commensal bacteria, indicating that POS act in a strain-dependent manner.

Finally, HMOs and XOS are able to inhibit the growth of *S. aureus* by exhibiting antibiofilm activity. Indeed, HMOs isolates from several donors significantly reduced biofilm production of MRSA, while the reductions ranged from 30 to 60% in comparison to the control (105). Moreover, XOS demonstrated antibacterial activity against S. aureus, not only by inhibiting biofilm formation, but also by affecting cell membrane permeability and obstructing Ca^{2+}-Mg^{2+}- ATPase activity on the cytomembrane of *S. aureus* (138).

***Clostridium* spp.**

Studies concerning the direct anti-pathogenic functionalities of NDOs against Clostridium spp. are very limited. To date, only FOS have been found to exert direct antimicrobial effects against *C. difficile*. More specifically, through an *in vitro* study FOS exhibited anti-adhesion potential towards several *C. difficile* strains on human epithelial cells (139). Although the underlying mechanism of these anti-adhesive properties was not investigated, it was speculated that FOS possibly affect the surface proteins and adhesins of the bacteria. Additionally, 8% FOS was found to significantly reduce *C. difficile* biofilm formation (139). The anti-biofilm effect of FOS may be correlated with its anti-adhesive effect since adhesion constitutes the primary step of colonization and biofilm formation.

NDOs can also fight indirectly against EPB, however since this is not the focus of the review, the indirect mechanisms will be shortly discussed in the next paragraph.

4.1.2. Indirect Mechanisms of Action of NDOs and SCFA against EPB

NDOs and SCFA can maintain gut homeostasis through indirect mechanisms based on microbiota-dependent effects, such as antimicrobial activity of beneficial bacteria, as well as microbiota-independent effects related to barrier-protecting and immune-related properties. Initially, NDOs and SCFA, as their metabolites, can stimulate the growth of beneficial bacteria, which subsequently interfere with the maintenance of gut homeostasis and finally decrease the pathogenic effect of EBP. The most abundant beneficial bacteria are *Bifidobacterium* and *Lactobacillus* species, while the major byproducts of oligosaccharide fermentation incorporated in the family of SCFA, are acetate (mainly produced by *bifidobacteria*), propionate (produced by *propionibacteria* and *Bacteroidetes*) and butyrate (mainly produced by *Lachnospiraceae* and *Ruminococcaceae*) (148). The defense role of the gut microbiota against EPB is based on several mechanisms, such as, antimicrobial activity and host immunity regulation induced by both beneficial bacteria and SCFA. Furthermore, gut microbiota acts against EPB by improving the intestinal barrier function of host cells and by reducing the luminal colonic pH due to the production of SCFA. Beyond the regulation of intestinal immunity and barrier function through microbiota-dependent effects, NDOs can promote gut immunity by their direct effects on specific immune and intestinal epithelial cells and also improve the intestinal barrier function by affecting

epithelial tight junction proteins and goblet cell function. Since the main focus of this review is based on the direct mechanisms of action of NDOs and SCFA against enterotoxins and EPB, the indirect mechanisms of action of NDOs and SCFA against EPB will be shortly discussed in this review.

Antimicrobial Activity of Beneficial Bacteria

NDOs promote the growth of beneficial bacteria, such as, *bifidobacteria* or *lactobacilli*, which in turn exert anti-pathogenic capabilities. Beneficial bacteria can also interfere with the adhesion of bacterial pathogens and exert antimicrobial activity by inhibiting their growth (149). FOS and inulin enhanced the antimicrobial activities of *Lactobacillus* spp. against pathogenic *S. aureus* and *E. coli*, an effect mostly related to the function of SCFA (acetate, propionate, isobutyric acid and butyrate) (110). An increase in the growth of *Lactobacillus* and *Bifidobacterium* spp. was also induced by XOS that additionally suppressed the growth of *C. perfringens*. Moreover, FOS supplementation in combination with five different probiotics provoked growth inhibition of *E. coli* and *C. difficile*, although no mechanism was determined (108). Finally, stimulation of different *Bifidobacterium* and *Lactobacillus* spp. growth by GOS and IMOS conferred protection against *C. difficile* infected mice (113).

Immunomodulation Activity

NDOs can exhibit anti-inflammatory effects that are likely to be driven by beneficial gut bacteria and their metabolites. Indeed, stimulation of *Bifidobacterium* growth by GOS, diminished the incidence of colitis leading to enhanced NK cell function and IL-15 production (150). Several beneficial bacteria such as different *Bifidobacterium* spp. were also found to increase the levels of IgA-producing cells in the lamina propria, therefore stimulating the secretion of sIgA into the luminal mucus layers and preventing the colonization of bacteria in the epithelium (151). Furthermore, via a cascade of signaling events, beneficial bacteria can promote the secretion and the production of anti-inflammatory cytokines, such as, IL-10 and TGFβ by T-regulatory cells (152). Additionally, FOS can reduce intestinal inflammation and colitis incidences, mediated by the induced growth of intestinal lactic acid bacteria in the colon (153).

In addition to the regulation of gut immunity through microbiota-dependent effects, NDOs can promote intestinal immunity by their direct effects on specific immune cells and intestinal epithelial cells. Indeed, several *in vitro* studies demonstrated the effects of NDOs on cytokine and chemokine production and release by different intestinal epithelial cell lines exposed to inflammatory triggers. For example, 2'-fucosyllactose inhibited the induction of IL-8 caused by different strains of *E. coli* in T84 cells (154), while GOS prevented the secretion of IL-8 in Caco-2 cells (155). Direct effects of NDOs on immune and epithelial cells are extensively reviewed by Yang et al. and Jeurink et al. (156,157).

Improvement of Intestinal Barrier Function

The disruption of epithelial barrier integrity constitutes one of the major pathological effects of EPB, and different studies describe that NDOs may substantially contribute to the protection of the epithelial barrier. Several NDOs improve intestinal epithelial barrier integrity by stimulating the growth of beneficial bacteria (158). Multiprotein complexes, termed as tight junctions (TJ), tightly connect epithelial cells to their neighbors in order to control paracellular permeability and transepithelial transport (159). FOS supplementation in mice decreased intestinal permeability and enhanced TJ integrity by promoting the growth of *Lactobacillus* and *Bifidobacterium* spp. (160). A potential cause underlying this effect is the control of intestinotrophic hormone glucagon-like peptide 2 production, a key element in the regulation of intestinal barrier secreted by endocrine L cells (160). Furthermore, in rats, fructans stimulated mucosa-associated *bifidobacteria* which was associated with increased mucus layer and improved mucosal architecture. Consequently, the increase in villus height and crypt depth, in addition to alterations in mucin composition resulted in gut mucosal barrier stabilization (161).

Additionally, SCFA can also contribute to the amelioration of the intestinal barrier. Butyrate is the preferential source of energy for colonic epithelial cells and the most potent acid among the SCFA (162). Improvement of intestinal epithelial barrier by butyrate likely relies on the expression of TJ proteins (163). Butyrate can accelerate the assembly of TJ by reorganizing TJ molecules, such as, ZO-1 and occludin, an effect mediated by the activation of AMP-activated protein kinase (AMPK) (163). In addition, SCFA enhance oxygen consumption by IECs resulting in a reduction in oxygen tension, leading to the stabilization of hypoxia-inducible factor (HIF). Indeed, butyrate increased barrier function and attenuated the infection of *C. difficile* infected mice through the stabilization of HIF-1 (164). SCFA can also exert intestinal protective mechanisms to the host by altering mucus production and secretion (165). Mucins are colonic mucous glycoproteins that promote a protective effect against toxic agents through the formation of a mucus layer that acts as physical barrier for the host. SCFA and especially, propionate and butyrate, can reinforce the mucus layer by stimulating mucin2 (MUC2) gene expression, which is the most prominent mucin on the intestinal mucosa surface [166]. The mechanisms that enable butyrate to be involved in MUC2 regulation are mediated via an active region (AP-1) within the MUC2 promotor and histone modifications (166).

In addition to the microbiota-dependent mechanisms for the improvement of the intestinal barrier, several studies have described the direct effects of NDOs on intestinal barrier function. 2' -fucosyllactose and lacto-N-neotetraose promoted enhanced barrier function by increasing the transepithelial resistance in Caco-2Bbe cells (167). GOS facilitated the tight junction assembly and stabilized the expression and the cellular distribution of the tight junction protein, claudin-3 (155). Additionally, GOS enhanced mucosal barrier function via the direct stimulation of goblet cells through the up-regulation of gene expression levels of secretory products and Golgi-sulfotransferases in a goblet cell line (168). Different NDOs

have a protective role on intestinal barrier function by differentially affecting epithelial tight junction proteins and goblet cell function, which has been reviewed previously (156).

Acidic Environment

SCFA production can lower the pH of the intraluminal space, creating an acidic environment that favors the growth of bifidogenic bacteria. In animal studies, co-administration of GOS with Bifidobacterium breve increased the anti-infectious activity against methicillin resistant *S. aureus* (MRSA), due to a high acetic acid production (112). Additionally, low intestinal pH and high concentration of acetic acid inhibited Stx production in STEC infected mice suggesting that such conditions create an unfavorable environment for bacterial pathogens (169). *E. coli* was also unable to survive inside the acidic environment in FOS- and XOS-fermented cultures leading to growth inhibition (111). Therefore, the more acidic environment created by SCFA constitutes an unfavorable space for pathogenic bacteria and subsequently inhibits their colonization.

4.2. The Effects of NDOs and SCFA against Bacterial Enterotoxins

The cytotoxicity of enterotoxins has been shown to be highly attenuated by the activity of NDOs and SCFA. A summary of their protective effects (both direct and indirect) against bacterial enterotoxins related to human diseases is provided in Table 4. Concerning NDOs, the majority of the mechanisms underlying their anti-virulent behavior rely on receptor mimicry mechanisms and their interference in endocytic pathways, such as the activation of the AMPK protein or the reduction in rRNA depurination. SCFA can also function against enterotoxins through several mechanisms, including metabolic integration, microbiota regulation, inhibition of fluid secretion, and maintenance of intestinal epithelial integrity. The absorption of SCFA in colonic epithelial cells, enable them to influence both extracellular and intracellular host compartments that might subsequently modulate the pathways activated by enterotoxins. However, to date, not all enterotoxins are shown to be inhibited by NDOs and SCFA.

4.2.1. *B. cereus* Enterotoxins

Characterized by their ability to form pores in epithelial cells, leading to fluid release and necrosis, B. cereus enterotoxins Hbl, Nhe and CytK do not necessarily need cellular receptors to express their pathogenicity. NDOs with high MW are incapable of crossing the cellular membrane, therefore their role is limited to the extracellular domains of host cells. This further justifies why the majority of therapeutic mechanisms of high MW NDOs focus on outer membrane cellular receptors. To date, no oligosaccharide treatment has been found against B. cereus-related enterotoxins, which is most probably due to the lack of cellular receptors and subsequently to the lack of receptor mimicry mechanism. Therapeutic activity against *B. cereus* enterotoxins has not been found either by SCFA. However, since SCFA have been proven to maintain fluid secretion (170), they can probably enhance the extensive influx of Ca^{2+}, Na^{+} and efflux of K^{+} caused by *B. cereus* enterotoxins.

4.2.2. Cholera Toxin (CT)

NDOs against CT

Several mechanisms contribute to the anti-virulence activity of NDOs against CT. First, COS can suppress intestinal fluid secretion, a key consequence underlying secretory diarrhea induced by CT (171). Indeed, luminal exposure to COS has been found to reduce intestinal fluid secretion in a mouse model by 30%, a reduction that relies on the activation of AMPK. AMPK is a heterotrimeric protein that apart from its role as a cellular energy conserver, can also mediate epithelial functions, such as tight junction assembly and ion transport (CFTR Cl– channel). COS of low MW (5000 Da) were found to interact with calcium-sensing receptor (CaSR), a Gq-coupled receptor linked to phospholipase C (PLC) located in IEC. Consequently, through a CaSR-PLC-IP3-receptor channel-dependent pathway, COS induce $Ca2^{+}$ secretion from ER and mitochondria, resulting in AMPK activation (171). Therefore, activated AMPK reduces CT-induced intestinal hypersecretion of chloride, highlighting the substantial anti-diarrheal activity of COS. Since overstimulation of fluid secretion is the consequence of multiple enterotoxins, COS might also be a potential therapy fighting against the adverse effects of other enterotoxins.

The second mechanism arises from direct interaction of NDOs with CT by competing with the GM1 receptor. GM1, the native receptor of CT, is a glycolipid receptor containing a sialylated carbohydrate structure. It is well known that galactose and N-acetylneuraminic acid have a substantial role in the majority of interactions between the receptor and the toxin, since removal of one of these residues confers loss of binding. Such components are also found in the structure of sialylated-oligosaccharides (SOS). In comparison with single monosaccharides, such as lactose, galactose and sialic acid that have been found to be ineffective inhibitors of CT-GM1 binding, the biantennary nature of the glycan chains in SOS showed increased potency for CT-GM1 inhibition (172). Additionally, 3' -sialyllactose, a predominant sialylated substance in human milk, which partially has the same sequence of the carbohydrate portion of GM1, was also found to behave as a receptor analogue for CT (173). In addition to acidic HMOs, GOS also contain saccharide residues (e.g., galactosyl residues) that pre-sent similarities with the GM1 structure and therefore showed inhibitory activity against CT binding to the GM1 receptor (174).

Rather than GM1, which is widely the sole receptor for CT intoxication, fucosylated glycan epitopes on glycoproteins were also found to facilitate cell surface binding and endocytic uptake of CT (175). Although, interaction of the CT binding subunit (CTB) with fucosylated glycans has a much lower affinity than the CTB-GM1 interaction, CTB binding studies demonstrated that low-affinity ligands can be recognized by CTB even in the presence of a much higher affinity ligand. Therefore, based on the functional significance of fucose recognition by CTB, fucosylated molecules can competitively interfere with CTB binding to intestinal epithelial cell lines and primary cells, to prevent CT uptake. Indeed, 2' -FL, a fraction identified also in HMOs mixtures, was shown to inhibit CTB binding to GM1 (176).

The appearance of additional binding sites is further justified by a study in which 20 of the most abundant HMOs were tested against CT and despite their affinity, the binding site was found to be distinct from the one of the native receptor binding sites on CT (177). Dendrimers, as obtained by synthetic conjugation of GM1-oligosaccharides, yielded very potent inhibitors of CTB with picomolar potencies (178) as evaluated by binding studies using intestinal organoids (179). Additionally, simplified polymeric ligands were shown to be very potent, even when incorporating fucose derivatives (180,181).

SCFA against CT

Cytotoxic consequences of CT such as watery diarrhea, are directly linked with low fluid absorption and hypersecretion of electrolytes and water in the intestinal lumen. SCFA can enhance the impairment of colonic functions occurring during CT pathogenicity by stimulating colonic absorption and reducing net fluid loss. Even if fluid secretion stimulated by CT mostly occurs from the small intestine, the colon can also bind with CT and secrete fluid and electrolytes after exposure to purified CT. An *in vivo* study showed that SCFA (acetate, propionate, butyrate) can significantly reduce the secretion of water and electrolytes (Na^+ , K^+ , Cl^-) in the colon of a CT-induced rabbit model. The anti-secretory behavior of SCFA is likely a result of their pro-absorptive effects on Na^+ and Cl^- transport. Interestingly, similar inhibitory effects of SCFA were not observed in the case of $HCO3^-$ secretion (182).

4.2.3. *C. difficile* Enterotoxins

NDOs against *C. difficile* Toxin A (TcdA) and Toxin B (TcdB)

Two substantial oligosaccharides, HMOs and FOS, show anti-virulent activity against *C. difficile* large toxins, TcdA and TcdB. Both enterotoxins incorporate two distinct functional regions in their structure, i.e., the CROPs and the region N-terminally adjacent to the CROPs, which independently serve as receptor-binding domains. Multiple receptors can bind TcdA and TcdB whereas glycans were identified as high-affinity binding structures for TcdA and specific protein receptors were identified for TcdB. However, both of them have carbohydrate-binding sites that bind to HMOs (183). Concerning TcdA, a variety of glycans, including the linear B type 2 trisaccharide α-Gal-(1,3)-β-Gal-(1,4)-β-GlcNAc, can bind either to TcdA or to a part of the TcdA CROPs (184). Therefore, HMOs that present structural similarities with the cellular receptors, can inhibit toxin binding. Such structures were found to be LNFPV and LNnH, two HMO structures, which demonstrated high binding affinity to TcdA, thereby obstructing toxin binding to its native receptor (185). Interestingly, molecular docking analysis of the two aforementioned compounds showed stronger binding to the TcdA binding site than that of α-Gal-(1,3)-β-Gal-(1,4)-β-GlcNAc, which further potentiates the role of HMOs in inhibiting toxin binding (185).

Additionally, FOS have protective effects against *C. difficile* infections by inhibiting the expression of toxin-related genes. FOS decreased the gene copy numbers of the Clostridium cluster XI and of the *C. difficile* toxin B (TcdB) in the fecal microbiota of rats in

an inflammatory bowel disease model, correlating with the reduction in chronic intestinal inflammation (186) and nosocomial diarrhea. The ability of FOS to exert anti-inflammatory effects possibly relies on the formation of SCFA, although further research is needed.

SCFA against *C. difficile* Toxin A (TcdA) and Toxin B (TcdB)

Multiple SCFA have been shown to efficiently reduce cytotoxicity of *C. difficile* enterotoxins, predominately via indirect pathways. Anti-pathogenic mechanisms of increased SCFA concentrations mostly rely on their ability to create an acidic luminal environment. Using *in vitro* experiments, the relationship between *C. difficile* enterotoxins' production with different concentrations of SCFA (acetate, propionate, butyrate) and pH levels was investigated. Results pointed out inhibitory effects of SCFA on the growth and the production of *C. difficile* enterotoxins, while beneficial effects were related to elevated SCFA concentration and lower pH levels (187). In addition, SCFA, and especially butyrate, exhibits protective effects against CDI by restoring the damage of IECs induced by *C. difficile* enterotoxins. A recent study was conducted to examine the mode of action of butyrate against CDI and revealed that even if no effects on bacterial colonization or *C. difficile* enterotoxin production was observed, butyrate managed to attenuate intestinal inflammation and improved the intestinal barrier function in CD-infected mice by acting directly on IECs. The reduction in intestinal epithelial permeability induced by butyrate was achieved via an HIF-1a-dependent mechanism. Administration of butyrate in mice infected with *C. difficile* demonstrated elevated levels of Hif1a expression and stability that is a relevant effect for intestinal barrier integrity. Through the stabilization of HIF-1, damage of IECs caused by *C. difficile* toxins, was repaired thereby preventing the local inflammatory response and systemic implications (164). In comparison with the large clostridial enterotoxins (TcdA, TcdB), so far, no effect has been reported by NDOs and SCFA against the CDT enterotoxin produced by *C. difficile*.

4.2.4. *C. perfringens* Enterotoxins

The binding of the two human related *C. perfringens* enterotoxins, CPE and CPB, to intestinal epithelial cells relies on the presence of tight and gap junctions. CPE binds strongly to claudin-3, -4 cellular receptors (188), while CPB binds to pannexin receptor (P2X7) that belongs to gap junction proteins, proteins that are responsible for intracellular communication (21). Dietary components, such as NDOs and their metabolites are known to regulate intestinal barrier function by changing the expression and the distribution of junction proteins (155,189). However, no interaction between these specific receptors and NDOs and SCFA has been identified so far.

On the other hand, SCFA have shown potential against CPE by inhibiting the spore formation stage. Unlike other enterotoxins that are produced and released during the replicative cycle of bacteria, CPE is induced during sporulation. SCFA can inhibit spore formation and subsequently enterotoxin production, thereby preventing associated undesirable consequences, such as antibiotic-associated diarrhea. Indeed, a study has shown that four

SCFA (acetate, isobutyrate, isovalerate, succinate) produced by *Bacteroides fragilis* can lower the amount of heat-resistant spores or even reduce the number of viable cells as observed for isobutyrate (190). Therefore, fermentation products of Bacteroides spp. can inhibit sporulation of *C. perfringens* and thus prevent CPE production.

4.2.5. Heat-Labile (LT) and Heat-Stable (ST) Enterotoxins

NDOs against LT and ST Enterotoxins

NDOs have shown anti-pathogenic functionalities against LT and ST enterotoxins, which are both produced by virulent *E. coli* strains. The protective role of HMOs and specifically of fucosylated oligosaccharides against ST enterotoxins has been identified by using a suckling mouse model (191). Additional studies that examine structurally similar HMOs proved similar effects against STa in T84 intestinal cells (192). The mechanism that potentially underlies this effect relies on the allosteric binding of HMOs to the STa receptor. Even if predominately NDOs bind directly to enterotoxins in order to inhibit their action, in the case of STa, fucosylated oligosaccharides bind preferentially to the STa receptor. Cytotoxicity of STa begins with the binding to GC-C that subsequently leads to the activation of the GC-C intracellular catalytic domain. Fucosylated fractions of human milk were found to block activation of human GC by binding allosterically to GC-C and therefore prevent STa infection (192)

HMOs can also bind directly to LT although at a distinct position of the native LT receptor (GM1). However, an evidence of competitive binding between GM1 and two HMOs (2'-fucosyllactose and lacto-N-fucopentaose I) gives the perspective of a receptor mimicry mechanism (177). Besides the direct effect of HMOs on LT, FOS can also interfere with the cytotoxicity of LT. By acting synergistically with *Lactobacillus rhamnosus*, FOS through an *in vitro* study were found to significantly inactivate ETEC by decreasing LT production (193). The reduction in the amount of enterotoxin is possibly an indirect effect of FOS that derives from the production of SCFA metabolites.

SCFA against LT Enterotoxin

SCFA can disturb the production of LT, however no mechanism has been determined so far. A study investigating the effect of SCFA on the production of LT enterotoxin showed that the addition of SCFA with different carbon chain length can significantly reduce or abolish LT production. In addition to the three main products of oligosaccharide fermentation, acetic (C-2), propionic (C-3) and butyric acid (C-4), three more SCFA presented inhibitory effects against LT, including *n*-valeric (C-5), *n*-caproic (C-6) and *n*-heptylic acids (C-7). The effectiveness of SCFA at a concentration of 2 mg/mL was proportional to the elongation of carbon chain length from C-2 to C-7. Eventually, *n*-heptylic acid (C-7) showed the most intense inhibition of LT production, while longer chain fatty acids tested (C-8 to C-10), inversely recovered the LT levels (194). Since production of LT is essential for the induction of diarrhea, by reducing the LT production, diarrheal consequences can be prevented.

4.2.6. Shiga Toxins (Stxs)

NDOs against Stxs

Specific NDOs such as POS and HMOs, have the capability to reduce Stx cytotoxicity. POS derived from the hydrolysis of citrus and apple pectin were found to completely protect human colonic HT29 cells from the toxic effects of Stx1 and Stx2 (195). The structure of POS is mostly composed of a GalA-rich backbone, a carbohydrate moiety similar, but distinct from the structure of the Gb3 receptor, thereby receptor mimicry may not be involved (140). However, POS can minimize Stx cytotoxicity by reducing rRNA depurination of host cells, an effect caused by the enzymatically active Stx A1 fragment that subsequently leads to protein synthesis inhibition (196). Indeed, reduction in rRNA depurination induced by POS was proved through a study that was based on a TaqMan probe-based RT-qPCR analysis (140). This further suggests that POS might block the entry of Stx into cells.

In addition to the relationship of Stxs with POS, a variety of HMOs have been shown to bind with several enterotoxins, including Stxs (177). Such potential of binding was further investigated and the measured binding affinities to HMOs were found to be lower in comparison with the affinity of Stxs to the Gb3 analogue, P^k trisaccharide. However, apparent association constants $K_{a,app}$ for HMOs binding to Stx, was found to be similar to many biologically relevant carbohydrate–protein interactions, therefore HMOs could compete with such monovalent interactions. Surprisingly, even if HMOs manage to bind efficiently with Stxs, competitive assays proved that the specific binding occurred at a distinct position rather than at the Gb3 binding site (177). Given these results, receptor binding inhibition seems unlikely to occur, therefore additional inhibitory mechanisms might be involved and further research on the anti-pathogenic effects of HMOs against Stxs should be established.

Table 4. Effects of NDOs and short chain fatty acids (SCFA) against bacterial enterotoxins.

Enterotoxins	NDOs/SCFA	Mechanism of Action	Health Benefit	Ref.
Cholera toxin	COS	Activation of AMPK	Reduction in CT-induced intestinal fluid secretion	(171)
Cholera toxin	SOS	Mimicking GM1 receptor	Anti-adhesive effect	(172)
Cholera toxin	Pooled HMOs	GM1 mimicking	Inhibition of sialyllactose on fluid accumulation induced by CT	(173)
Cholera toxin	GOS	Mimicking GM1 receptor	Anti-adhesive effect	(174)
Cholera toxin	2'-FL	2'-FL resembles fucosylated glycan epitopes	Inhibition of CTB binding	(176)
Cholera Toxin	2'FL, LNnT, LNFP I, II, III	Binding with CTB	No inhibitory effect	(177)
Cholera toxin	Acetate, propionate, butyrate	Reduction in water and electrolyte secretion	Anti-diarrheal effects	(182)
Shiga toxins	2'FL, LNnT, LNFP I, II, III	Binding with STX	No inhibitory effect	(177)
Shiga toxins	POS	POS mimic the interaction with the galabiose receptor	Inhibition of Stx	(195)
Shiga toxins	POS	Reduction in rRNA depurination	Reduction in Stx cytotoxicity	(140)
Shiga toxins	Acetic acid, Lactic acid	Decrease in pH	Reduction in stx2 gene expression	(169,197)
Shiga Toxins	Acetate	Reduction in transepithelial electrical resistance	Prevention of Stx translocation	(198,199)
Heat-stable enterotoxin (ST)	Fucosylated HMOs	not determined	Protective effect (suckling mice)	(191)
Heat-stable enterotoxin (ST)	Pooled HMOs, Fucosylated HMOs	Block of human guanylate cyclase activation by allosteric binding to the STa receptor	Protective effect (T84 cells)	(192)
Heat-labile enterotoxin (LT)	FOS Bifidogenic effect with L. rhamnosus	Reduction in growth and toxin	production	(193)

Table 4. Continued

Enterotoxins	NDOs/SCFA	Mechanism of Action	Health Benefit	Ref.
Heat-labile enterotoxin type 1 (LT-1)	2'FL, LNnT, LNFP I, II, III	Binding with LTB	No inhibitory effect	(177)
Heat-labile enterotoxin (LT)	Acetic, propionic, butyric, valeric, caproic and heptylic acids	Effects on the biosynthesis of LT	Reduction in LT production	(194)
C. difficile toxin B (TcdB)	FOS, inulin	Inhibiting toxin-related gene expression	Anti-inflammatory effect	(186)
C. difficile toxins A (TcdA) and B (TcdB)	Pooled HMOs	Mimicking the structure of cellular receptors	Inhibition of toxins' binding to cellular receptors	(185)
C. difficile toxins A (TcdA) and B (TcdB)	Acetate, propionate, butyrate	Decrease in pH	Prevention of growth and elaboration of toxin (colonization resistance)	(187)
C. difficile toxins A (TcdA) and B (TcdB)	Butyrate	Stabilization of HIF-1	Anti-inflammatory effects and intestinal barrier function improvement	(164)
C. perfringens enterotoxin	Acetate, isobutyrate, isovalerate, succinate	Inhibition of toxin sporulation	Reduction in enterotoxin cytotoxicity	(190)

4.2.7. Staphylococcal Enterotoxins (SEs)

To date, to the best of our knowledge, there is no evidence of inhibitory effects of NDOs against SEs. The cytotoxicity pathway of SEs starts with their binding to MHC II class molecules after crossing the epithelial barrier. Binding of SEs to MHC II class constitutes a pivotal step for their cytotoxicity and is one of the main therapeutic targets. The inactivity of NDOs against SEs might be related to the difficulty of high MW NDOs to cross the epithelial barrier, leading to a diminished chance for interaction with intracellular targets such as MHC II class. On the other hand, SCFA and especially butyrate is a well-known HDAC inhibitor and thus can downregulate pro-inflammatory mediator expression leading to increased regulatory T cell differentiation (201,202) However, even if SCFA have been proved to modulate host immune responses, no effect of SCFA was reported on MHC class II (203).

5. Conclusions and Future Perspectives

This review presents an overview of a great number of NDOs and SCFA, as their fermentation products produced by the gut microbiota, that could be considered as therapeutic agents to limit the cytotoxic effects on the human intestine induced by EPB and their toxins. Enterotoxins can drastically damage intestinal epithelial cells, leading to gastrointestinal and systematic complications. To exert their cytotoxicity, enterotoxins mostly alter cell viability through the inactivation or the cleavage of intracellular targets or by promoting the efflux of water and electrolytes through the formation of membrane pores. A thorough exploration of the key characteristics of intestinal pathogens and their toxins, resulted in the selection of various NDOs and SCFA as potential treatments fighting against EPB-related infections.

NDOs interact with EPB and thereby inhibit their activity following either direct or indirect mechanisms. Direct effects of NDOs on EPB, including anti-adhesive, antibiofilm and anti-growth effects, partly rely on receptor mimicry strategies. The structural similarity between adhesive receptors of EPB and free oligosaccharides, enable NDOs to mimic bacterial binding to host cell surface, thereby blocking bacterial adherence. The anti-microbial potential of NDOs is not only governed by their structural similarity with the receptors, since other characteristics, such as e.g., their charge also endows them with additional potency to interact with EPB. However, not all related studies proposed a thorough explanation for the direct anti-pathogenic effects of NDOs. Consequently, a more elucidative characterization of NDOs along with a wider range of pathogens, could reveal further details of their anti-microbial capabilities. On the other hand, indirect strategies of NDOs, rely on the maintenance of gut homeostasis, mediated by the promotion of beneficial microbiome and subsequently by the activity of SCFA. Additionally, barrier-protecting and immune-related properties of NDOs and SCFA can also contribute to the maintenance of gut homeostasis.

Except the indirect and direct anti-pathogenic effects of NDOs on EPB, NDOs and SCFA influence the cytotoxicity of EPB-associated enterotoxins. The anti-toxin activity of NDOs

mostly derives from the blockage of the enterotoxin adherence, as the primary step of the toxin-induced cytotoxicity pathway. Structural similarity between NDOs and toxin receptors leads to inhibition of toxin adherence. Despite their effects on the extracellular compartments, LMW NDOs have been shown to influence intracellular enterotoxin pathways, leading to the reduction in fluid hypersecretion. Similar anti-toxin potentials are also identified by SCFA, since their low MW enable them to cross the intestinal epithelium barrier and thereby target intracellular targets (e.g., HIF-1 factor). However, the most prominent cause of anti-toxin effects induced by SCFA is the creation of an acidic environment that results in unfavorable conditions for the enterotoxins. To uncover additional targets towards the pathways of enterotoxins, further research is warranted to elucidate adhesive mechanisms and eventually lead to the optimal NDOs or SCFA treatment corresponding to specific enterotoxin mechanisms.

Based on the findings of this review, NDOs (e.g., HMOs, GOS and POS) that acquire the highest structural resemblance with host cells receptors for enterotoxins demonstrated the highest anti-pathogenic capacity. Given the promising anti-pathogenic potential of NDOs, further research related to appropriate utilization of NDOs and SCFA through a clinical approach is required. Although food consumption and production vary between cultures, regions, and countries, NDOs are present in a wide variety of sources found in the regular human diet (204). Different individual factors, like metabolism, gender, age, genetic variations and microbiome composition will influence the response to these dietary interventions, therefore, it is complicated to suggest a general sufficient amount and source of NDOs. In this regard, personalized nutrition will be more suitable than population-based nutritional advice (205). NDOs are mostly tested as individual agents, but it could be interesting to evaluate the combination of NDOs with different SCFA to indicate if there is a possibility to exert a synergistic effect. To date, antibiotics are considered as the major therapeutic strategy to address infectious diseases. However, the improper and high use of antibiotics result in a decreased susceptibility and increased resistance against these antimicrobials. The use of NDOs and SCFA in lieu of or in combination with antibiotics to control infectious diseases might contribute to a reduction in this emerging antibiotic resistance. Additionally, several NDOs (such as GOS, FOS) have been already added in infant nutrition formulas in an attempt to mimic the endogenous HMOs. Thereby, NDOs already offer a safer and non-toxic alternative for anti-microbial therapy. In this respect, NDOs and SCFA provide specific, targeted activity and are less likely to present negative side effects in comparison with commensal treatments such as antibiotics. Given the burden and long-term consequences of microbial-associated gastrointestinal diseases, the route of using NDOs and SCFA to fortify gut flora and encounter EPB and their toxins holds much potential. Therefore, we anticipate that with additional focused studies, these anti-toxin molecules could soon reach the optimal goal to be used as therapeutic agents with great impact on the treatment of infectious diseases.

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Conceptualization, M.A. and G.-N.I.; writing—original draft preparation, G.-N.I. and M.A.; writing—review and editing, M.A. and P.A.J.H. and R.P. and G.F. and S.B.; Table and Figure creation, G.-N.I. and M.A. All authors have read and agreed to the published version of the manuscript

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Abebe E, Gugsa G, Ahmed M. Review on major food-borne zoonotic bacterial pathogens. J Trop Med. 2020;2020.
2. Mussatto SI, Mancilha IM. Non-digestible oligosaccharides: A review. Carbohydr Polym. 2007;68(3):587–97.
3. Singh P. Functional Oligosaccharides: Physico-chemical Properties, Synthesis, Structures and Biological Applications. JICS. 2019;9:674–90.
4. Libertucci J, Young VB. The role of the microbiota in infectious diseases. Nature Microbiology. 2019;4(1):35–45.
5. Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nature Reviews Immunology. 2008;8(6):411–20.
6. Popoff MR. Multifaceted interactions of bacterial toxins with the gastrointestinal mucosa. Future Microbiology. 2011;6(7):763–97.
7. Aslam B, Wang W, Arshad MI, Khurshid M, Muzammil S, Rasool MH, et al. Antibiotic resistance: a rundown of a global crisis. Infect Drug Resist. 2018;11:1645.
8. Macfarlane S. Antibiotic treatments and microbes in the gut. Environ Microbiol. 2014;16(4):919–24.
9. Akbari P, Fink-Gremmels J, Willems RHAM, Difilippo E, Schols HA, Schoterman MHC, et al. Characterizing microbiota-independent effects of oligosaccharides on intestinal epithelial cells: Insight into the role of structure and size. Eur J Nutr. 2017;56(5):1919–30.
10. Patel S, Goyal A. Functional oligosaccharides: production, properties and applications. World Journal of Microbiology and Biotechnology. 2011;27(5):1119–28.
11. Sun Y, O'Riordan MXD. Regulation of bacterial pathogenesis by intestinal short-chain fatty acids. Adv Appl Microbiol. 2013;85:93–118.
12. Jessberger N, Dietrich R, Granum PE, Märtlbauer E. The Bacillus cereus food infection as multifactorial process. Toxins (Basel). 2020;12(11):701.
13. Granum PE, Lund T. Bacillus cereus and its food poisoning toxins. FEMS Microbiol Lett. 1997;157(2):223–8.
14. Lund T, de Buyser M, Granum PE. A new cytotoxin from Bacillus cereus that may cause necrotic enteritis. Mol Microbiol. 2000;38(2):254–61.
15. Messelhäußer U, Ehling-Schulz M. Bacillus cereus—a multifaceted opportunistic pathogen. Current Clinical Microbiology Reports. 2018;5(2):120–5.
16. Senesi S, Ghelardi E. Production, secretion and biological activity of Bacillus cereus enterotoxins. Toxins (Basel). 2010;2(7):1690–703.
17. Stenfors Arnesen LP, Fagerlund A, Granum PE. From soil to gut: Bacillus cereus and its food poisoning toxins. FEMS Microbiol Rev. 2008;32(4):579–606.
18. Cui Y, Märtlbauer E, Dietrich R, Luo H, Ding S, Zhu K. Multifaceted toxin profile, an approach toward a better understanding of probiotic Bacillus cereus. Critical Reviews in Toxicology. 2019;49(4):342–56.
19. Sastalla I, Fattah R, Coppage N, Nandy P, Crown D, Pomerantsev AP, et al. The Bacillus cereus Hbl and Nhe tripartite enterotoxin components assemble sequentially on the surface of target cells and are not interchangeable. PLoS One. 2013;8(10):e76955.
20. Uzal FA, Freedman JC, Shrestha A, Theoret JR, Garcia J, Awad MM, et al. Towards an understanding of the role of Clostridium perfringens toxins in human and animal disease. Future Microbiol. 2014;9(3):361–77.
21. Navarro MA, McClane BA, Uzal FA. Mechanisms of action and cell death associated with Clostridium perfringens toxins. Toxins (Basel). 2018;10(5):212.
22. Nagahama M, Ochi S, Oda M, Miyamoto K, Takehara M, Kobayashi K. Recent insights into Clostridium perfringens beta-toxin. Toxins (Basel). 2015;7(2):396–406.
23. Fernandez Miyakawa ME, Redondo LM. Role of Clostridium perfringens Alpha, Beta, Epsilon and Iota toxins in Enterotoxemia of monogastrics and Ruminants. 2016;

24. Theoret JR, Uzal FA, McClane BA. Identification and characterization of Clostridium perfringens beta toxin variants with differing trypsin sensitivity and *in vitro* cytotoxicity activity. Infect Immun. 2015;83(4):1477–86.
25. Scallan E, Hoekstra RM, Angulo FJ, Tauxe R v, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis. 2011;17(1):7.
26. Kiu R, Hall LJ. An update on the human and animal enteric pathogen Clostridium perfringens. Emerg Microbes Infect. 2018;7(1):1–15.
27. Freedman JC, Shrestha A, McClane BA. Clostridium perfringens enterotoxin: action, genetics, and translational applications. Toxins (Basel). 2016;8(3):73.
28. Günzel D, Yu ASL. Claudins and the modulation of tight junction permeability. Physiol Rev. 2013;93(2):525–69.
29. Robertson SL, Smedley III JG, Singh U, Chakrabarti G, van Itallie CM, Anderson JM, et al. Compositional and stoichiometric analysis of Clostridium perfringens enterotoxin complexes in Caco-2 cells and claudin 4 fibroblast transfectants. Cell Microbiol. 2007;9(11):2734–55.
30. Chen J, Theoret JR, Shrestha A, Smedley III JG, McClane BA. Cysteine-scanning mutagenesis supports the importance of Clostridium perfringens enterotoxin amino acids 80 to 106 for membrane insertion and pore formation. Infect Immun. 2012;80(12):4078–88.
31. Bookshelf N. StatPearls. Staphylococcus Aureus. Available online: https://www ncbi nlm nih gov/books/NBK441868/(accessed on 18 November 2020).
32. Pinchuk I v, Beswick EJ, Reyes VE. Staphylococcal enterotoxins. Toxins (Basel). 2010;2(8):2177–97.
33. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, et al. Food-related illness and death in the United States. Emerg Infect Dis. 1999;5(5):607.
34. Scherrer D, Corti S, Muehlherr JE, Zweifel C, Stephan R. Phenotypic and genotypic characteristics of Staphylococcus aureus isolates from raw bulk-tank milk samples of goats and sheep. Veterinary Microbiology. 2004;101(2):101–7.
35. Benkerroum N. Staphylococcal enterotoxins and enterotoxin-like toxins with special reference to dairy products: An overview. Crit Rev Food Sci Nutr. 2018;58(12):1943–70.
36. Krakauer T. Staphylococcal superantigens: pyrogenic toxins induce toxic shock. Toxins (Basel). 2019;11(3):178.
37. Chandrasekaran R, Lacy DB. The role of toxins in Clostridium difficile infection. FEMS Microbiol Rev. 2017;41(6):723–50.
38. Martin JSH, Monaghan TM, Wilcox MH. Clostridium difficile infection: epidemiology, diagnosis and understanding transmission. Nature reviews Gastroenterology & hepatology. 2016;13(4):206–16.
39. Davies KA, Longshaw CM, Davis GL, Bouza E, Barbut F, Barna Z, et al. Underdiagnosis of Clostridium difficile across Europe: the European, multicentre, prospective, biannual, point-prevalence study of Clostridium difficile infection in hospitalised patients with diarrhoea (EUCLID). The Lancet infectious diseases. 2014;14(12):1208–19.
40. Feuerstadt P, Stong L, Dahdal DN, Sacks N, Lang K, Nelson WW. Healthcare resource utilization and direct medical costs associated with index and recurrent Clostridioides difficile infection: a real-world data analysis. Journal of Medical Economics. 2020;23(6):603–9.
41. Voth DE, Ballard JD. Clostridium difficile toxins: mechanism of action and role in disease. Clin Microbiol Rev. 2005;18(2):247–63.
42. Aktories K, Schwan C, Jank T. Clostridium difficile toxin biology. Annu Rev Microbiol. 2017;71:281–307.
43. Pothoulakis C, Galili U, Castagliuolo I, Kelly CP, Nikulasson S, Dudeja PK, et al. A human antibody binds to alpha-galactose receptors and mimics the effects of Clostridium difficile toxin A in rat colon. Gastroenterology. 1996;110(6):1704–12.
44. Na X, Kim H, Moyer MP, Pothoulakis C, LaMont JT. gp96 is a human colonocyte plasma membrane binding protein for Clostridium difficile toxin A. Infect Immun. 2008;76(7):2862–71.
45. Yuan P, Zhang H, Cai C, Zhu S, Zhou Y, Yang X, et al. Chondroitin sulfate proteoglycan 4 functions as the cellular receptor for Clostridium difficile toxin B. Cell Res. 2015;25(2):157–68.

46. LaFrance ME, Farrow MA, Chandrasekaran R, Sheng J, Rubin DH, Lacy DB. Identification of an epithelial cell receptor responsible for Clostridium difficile TcdB-induced cytotoxicity. Proceedings of the National Academy of Sciences. 2015;112(22):7073–8.
47. Chandrasekaran R, Kenworthy AK, Lacy DB. Clostridium difficile toxin A undergoes clathrin-independent, PACSIN2-dependent endocytosis. PLoS Pathog. 2016;12(12):e1006070.
48. Papatheodorou P, Zamboglou C, Genisyuerek S, Guttenberg G, Aktories K. Clostridial glucosylating toxins enter cells via clathrin-mediated endocytosis. PLoS One. 2010;5(5):e10673.
49. Genisyuerek S, Papatheodorou P, Guttenberg G, Schubert R, Benz R, Aktories K. Structural determinants for membrane insertion, pore formation and translocation of Clostridium difficile toxin B. Mol Microbiol. 2011;79(6):1643–54.
50. Irvine RF, Schell MJ. Back in the water: the return of the inositol phosphates. Nature reviews Molecular cell biology. 2001;2(5):327–38.
51. Gerding DN, Johnson S, Rupnik M, Aktories K. Clostridium difficile binary toxin CDT: mechanism, epidemiology, and potential clinical importance. Gut Microbes. 2014;5(1):15–27.
52. Papatheodorou P, Carette JE, Bell GW, Schwan C, Guttenberg G, Brummelkamp TR, et al. Lipolysis-stimulated lipoprotein receptor (LSR) is the host receptor for the binary toxin Clostridium difficile transferase (CDT). Proceedings of the National Academy of Sciences. 2011;108(39):16422–7.
53. Mesli S, Javorschi S, Berard AM, Landry M, Priddle H, Kivlichan D, et al. Distribution of the lipolysis stimulated receptor in adult and embryonic murine tissues and lethality of LSR−/− embryos at 12.5 to 14.5 days of gestation. Eur J Biochem. 2004;271(15):3103–14.
54. Ernst K, Schmid J, Beck M, Hägele M, Hohwieler M, Hauff P, et al. Hsp70 facilitates trans-membrane transport of bacterial ADP-ribosylating toxins into the cytosol of mammalian cells. Sci Rep. 2017;7(1):1–16.
55. Schwan C, Stecher B, Tzivelekidis T, van Ham M, Rohde M, Hardt WD, et al. Clostridium difficile toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. PLoS Pathog. 2009;5(10):e1000626.
56. Tang YW, Sails A. Molecular medical microbiology. Academic press; 2014.
57. Awofeso N, Aldbak K. Cholera, migration, and global health–a critical review. International Journal of Travel Medicine and Global Health. 2018;6(3):92–9.
58. Kuna A, Gajewski M. Cholera—the new strike of an old foe. Int Marit Health. 2017;68(3):163–7.
59. Ramamurthy T, Mutreja A, Weill FX, Das B, Ghosh A, Nair GB. Revisiting the global epidemiology of cholera in conjunction with the genomics of Vibrio cholerae. Front Public Health. 2019;203.
60. Organization WH. Cholera, 2012= Choléra, 2012. Weekly Epidemiological Record= Relevé épidémiologique hebdomadaire. 2013;88(31):321–34.
61. Keya C, Chatterjee SN. Cholera Toxins. Springer: Berlin/Heidelberg, Germany; 2009.
62. Sánchez J, Holmgren J. Cholera toxin—a foe & a friend. Indian J Med Res. 2011;133(2):153.
63. Aureli M, Mauri L, Ciampa MG, Prinetti A, Toffano G, Secchieri C, et al. GM1 ganglioside: past studies and future potential. Mol Neurobiol. 2016;53(3):1824–42.
64. Merritt EA, Sarfaty S, Akker F van den, L'Hoir C, Martial JA, Hol WGJ. Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. Protein Science. 1994;3(2):166–75.
65. Chinnapen DJF, Chinnapen H, Saslowsky D, Lencer WI. Rafting with cholera toxin: endocytosis and trafficking from plasma membrane to ER. FEMS Microbiol Lett. 2007;266(2):129–37.
66. Orlandi PA, Fishman PH. Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. J Cell Biol. 1998;141(4):905–15.
67. Torgersen ML, Skretting G, van Deurs B, Sandvig K. Internalization of cholera toxin by different endocytic mechanisms. J Cell Sci. 2001;114(20):3737–47.
68. Kirkham M, Fujita A, Chadda R, Nixon SJ, Kurzchalia T v, Sharma DK, et al. Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. J Cell Biol. 2005;168(3):465–76.
69. Wernick NLB, Chinnapen DJF, Cho JA, Lencer WI. Cholera toxin: an intracellular journey into the cytosol by way of the endoplasmic reticulum. Toxins (Basel). 2010;2(3):310–25.
70. Raufman JP. Cholera 12. Am J Med. 1998;104(4):386–94.

71. Denamur E, Clermont O, Bonacorsi S, Gordon D. The population genetics of pathogenic Escherichia coli. Nature Reviews Microbiology. 2021;19(1):37–54.
72. Duan Q, Xia P, Nandre R, Zhang W, Zhu G. Review of newly identified functions associated with the heat-labile toxin of enterotoxigenic Escherichia coli. Front Cell Infect Microbiol. 2019;292.
73. Jobling MG, Holmes RK. Heat-Labile Enterotoxins. EcoSal Plus. 2006;2(1).
74. Wang H, Zhong Z, Luo Y, Cox E, Devriendt B. Heat-stable enterotoxins of enterotoxigenic Escherichia coli and their impact on host immunity. Toxins (Basel). 2019;11(1):24.
75. Mudrak B, Kuehn MJ. Heat-labile enterotoxin: beyond G M1 binding. Toxins (Basel). 2010;2(6):1445–70.
76. Taxt A, Aasland R, Sommerfelt H, Nataro J, Puntervoll P. Heat-stable enterotoxin of enterotoxigenic Escherichia coli as a vaccine target. Infect Immun. 2010;78(5):1824–31.
77. Albano F, Thompson MR, Orrú S, Scaloni A, Musetta A, Pucci P, et al. Structural and functional features of modified heat-stable toxins produced by enteropathogenic Klebsiella cells. Pediatr Res. 2000;48(5):685–90.
78. Jackson MP, Neill RJ, O'Brien AD, Holmes RK, Newland JW. Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from Escherichia coli 933. FEMS Microbiology Letters. 1987;44(1):109–14.
79. Bryan A, Youngster I, McAdam AJ. Shiga toxin producing Escherichia coli. Clin Lab Med. 2015;35(2):247–72.
80. Majowicz SE, Scallan E, Jones-Bitton A, Sargeant JM, Stapleton J, Angulo FJ, et al. Global incidence of human Shiga toxin–producing Escherichia coli infections and deaths: a systematic review and knowledge synthesis. Foodborne Pathog Dis. 2014;11(6):447–55.
81. Melton-Celsa AR. Shiga toxin (Stx) classification, structure, and function. Microbiol Spectr. 2014;2(4):2–4.
82. Licznerska K, Nejman-Faleńczyk B, Bloch S, Dydecka A, Topka G, Gąsior T, et al. Oxidative stress in Shiga toxin production by enterohemorrhagic Escherichia coli. Oxidative Medicine and Cellular Longevity. 2016;2016.
83. Chan YS, Ng TB. Shiga toxins: from structure and mechanism to applications. Appl Microbiol Biotechnol. 2016;100(4):1597–610.
84. Lukyanenko V, Malyukova I, Hubbard A, Delannoy M, Boedeker E, Zhu C, et al. Enterohemorrhagic Escherichia coli infection stimulates Shiga toxin 1 macropinocytosis and transcytosis across intestinal epithelial cells. American Journal of Physiology-Cell Physiology. 2011;301(5):C1140–9.
85. Malyukova I, Murray KF, Zhu C, Boedeker E, Kane A, Patterson K, et al. Macropinocytosis in Shiga toxin 1 uptake by human intestinal epithelial cells and transcellular transcytosis. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2009;296(1):G78–92.
86. Sandvig K, Garred Ø, Prydz K, Kozlov J v, Hansen SH, van Deurs B. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. Nature. 1992;358(6386):510–2.
87. Joseph A, Cointe A, Mariani Kurkdjian P, Rafat C, Hertig A. Shiga toxin-associated hemolytic uremic syndrome: A narrative review. Toxins (Basel). 2020;12(2):67.
88. Wang Y, Guo Q, Goff HD, LaPointe G. Oligosaccharides: structure, function and application. 2019;
89. Seeberger PH. Monosaccharide diversity. 2017;
90. Liu J, Yang S, Li X, Yan Q, Reaney MJT, Jiang Z. Alginate oligosaccharides: production, biological activities, and potential applications. Comprehensive Reviews in Food Science and Food Safety. 2019;18(6):1859–81.
91. Kaczmarek MB, Struszczyk-Swita K, Li X, Szczęsna-Antczak M, Daroch M. Enzymatic modifications of chitin, chitosan, and chitooligosaccharides. Front Bioeng Biotechnol. 2019;243.
92. Muanprasat C, Chatsudthipong V. Chitosan oligosaccharide: Biological activities and potential therapeutic applications. Pharmacol Ther. 2017;170:80–97.
93. Ibrahim OO. Functional oligo-saccharides: chemicals structure, manufacturing, health benefits, applications and regulations. J Food Chem Nanotechnol. 2018;4:65–76.
94. Louis P, Flint HJ, Michel C. How to manipulate the microbiota: prebiotics. Microbiota of the human body. 2016;119–42.

95. Aparecida A, Tomal B, Farinazzo FS, Bachega A, Bosso A, Bonifácio Da Silva J, et al. Human Health Implications. Int J Nutr Food Sci. 2019;9.
96. Davani-Davari D, Negahdaripour M, Karimzadeh I, Seifan M, Mohkam M, Masoumi SJ, et al. Prebiotics: definition, types, sources, mechanisms, and clinical applications. Foods. 2019;8(3):92.
97. Boehm G, Stahl B, Jelinek J, Knol J, Miniello V, Moro GE. Prebiotic carbohydrates in human milk and formulas. Acta Paediatrica. 2005;94:18–21.
98. Wiciński M, Sawicka E, Gębalski J, Kubiak K, Malinowski B. Human milk oligosaccharides: Health benefits, potential applications in infant formulas, and pharmacology. Nutrients. 2020;12(1):266.
99. Hoving LR, van der Zande HJP, Pronk A, Guigas B, Willems van Dijk K, van Harmelen V. Dietary yeast-derived mannan oligosaccharides have immune-modulatory properties but do not improve high fat diet-induced obesity and glucose intolerance. Plos one. 2018;13(5):e0196165.
100. Liu XY, Wang Q, Cui SW, Liu HZ. A new isolation method of β-D-glucans from spent yeast Saccharomyces cerevisiae. Food Hydrocolloids. 2008;22(2):239–47.
101. Baldassarre S, Babbar N, van Roy S, Dejonghe W, Maesen M, Sforza S, et al. Continuous production of pectic oligosaccharides from onion skins with an enzyme membrane reactor. Food Chem. 2018;267:101–10.
102. Babbar N, Dejonghe W, Gatti M, Sforza S, Elst K. Pectic oligosaccharides from agricultural by-products: production, characterization and health benefits. Critical Reviews in Biotechnology. 2016;36(4):594–606.
103. Lin SH, Chou LM, Chien YW, Chang JS, Lin CI. Prebiotic effects of xylooligosaccharides on the improvement of microbiota balance in human subjects. Gastroenterol Res Pract. 2016;2016.
104. Aachary AA, Prapulla SG. Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties, and applications. Comprehensive Reviews in Food Science and Food Safety. 2011;10(1):2–16.
105. Ackerman DL, Craft KM, Doster RS, Weitkamp JH, Aronoff DM, Gaddy JA, et al. Antimicrobial and antibiofilm activity of human milk oligosaccharides against *Streptococcus* agalactiae, Staphylococcus aureus, and Acinetobacter baumannii. ACS Infect Dis. 2017;4(3):315–24.
106. Asadpoor M, Peeters C, Henricks PAJ, Varasteh S, Pieters RJ, Folkerts G, et al. Anti-pathogenic functions of non-digestible oligosaccharides *in vitro*. Nutrients. 2020;12(6):1789.
107. Ríos-Covián D, Ruas-Madiedo P, Margolles A, Gueimonde M, de Los Reyes-gavilán CG, Salazar N. Intestinal short chain fatty acids and their link with diet and human health. Front Microbiol. 2016;7:185.
108. Piatek J, Krauss H, Ciechelska-Rybarczyk A, Bernatek M, Wojtyla-Buciora P, Sommermeyer H. In-vitro growth inhibition of bacterial pathogens by probiotics and a synbiotic: product composition matters. International Journal of Environmental Research and Public Health. 2020;17(9):3332.
109. Anand S, Mandal S, Singh KS, Patil P, Tomar SK. Synbiotic combination of Lactobacillus rhamnosus NCDC 298 and short chain fructooligosaccharides prevents enterotoxigenic Escherichia coli infection. LWT. 2018;98:329–34.
110. Stefania D, Miranda P, Diana M, Claudia Z, Rita P, Donatella P. Antibiofilm and antiadhesive activities of different synbiotics. Journal of Probiotics and Health. 2017;5(3):182–91.
111. Fooks LJ, Gibson GR. *In vitro* investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. FEMS Microbiology Ecology. 2002;39(1):67–75.
112. Lkhagvadorj E, Nagata S, Wada M, Bian L, Wang C, Chiba Y, et al. Anti-infectious activity of synbiotics in a novel mouse model of methicillin-resistant Staphylococcus aureus infection. Microbiol Immunol. 2010;54(5):265–75.
113. Kondepudi KK, Ambalam P, Karagin PH, Nilsson I, Wadström T, Ljungh Å. A novel multi-strain probiotic and synbiotic supplement for prevention of Clostridium difficile infection in a murine model. Microbiol Immunol. 2014;58(10):552–8.
114. Martín-Sosa S, Martín MJ, Hueso P. The sialylated fraction of milk oligosaccharides is partially responsible for binding to enterotoxigenic and uropathogenic Escherichia coli human strains. J Nutr. 2002;132(10):3067–72.

115. Cravioto A, Tello A, Villafan H, Ruiz J, del Vedovo S, Neeser JR. Inhibition of localized adhesion of enteropathogenic Escherichia coli to HEp-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk. Journal of Infectious Diseases. 1991;163(6):1247–55.
116. Coppa G v, Zampini L, Galeazzi T, Facinelli B, Ferrante L, Capretti R, et al. Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: Escherichia coli, Vibrio cholerae, and Salmonella fyris. Pediatr Res. 2006;59(3):377–82.
117. Weichert S, Jennewein S, Hüfner E, Weiss C, Borkowski J, Putze J, et al. Bioengineered 2′-fucosyllactose and 3-fucosyllactose inhibit the adhesion of Pseudomonas aeruginosa and enteric pathogens to human intestinal and respiratory cell lines. Nutrition research. 2013;33(10):831–8.
118. Facinelli B, Marini E, Magi G, Zampini L, Santoro L, Catassi C, et al. Breast milk oligosaccharides: effects of 2′-fucosyllactose and 6′-sialyllactose on the adhesion of Escherichia coli and Salmonella fyris to Caco-2 cells. The Journal of Maternal-Fetal & Neonatal Medicine. 2019;32(17):2950–2.
119. Manthey CF, Autran CA, Eckmann L, Bode L. Human milk oligosaccharides protect against enteropathogenic Escherichia coli attachment *in vitro* and EPEC colonization in suckling mice. Journal of Pediatric Gastroenterology and Nutrition. 2014;58(2):165–8.
120. Khan S, Tøndervik A, Sletta H, Klinkenberg G, Emanuel C, Onsøyen E, et al. Overcoming drug resistance with alginate oligosaccharides able to potentiate the action of selected antibiotics. Antimicrob Agents Chemother. 2012;56(10):5134–41.
121. Fernandes JC, Tavaria FK, Soares JC, Ramos ÓS, Monteiro MJ, Pintado ME, et al. Antimicrobial effects of chitosans and chitooligosaccharides, upon Staphylococcus aureus and Escherichia coli, in food model systems. Food Microbiology. 2008;25(7):922–8.
122. Rhoades J, Gibson G, Formentin K, Beer M, Rastall R. Inhibition of the adhesion of enteropathogenic Escherichia coli strains to HT-29 cells in culture by chito-oligosaccharides. Carbohydr Polym. 2006;64(1):57–9.
123. Jeon YJ, Park PJ, Kim SK. Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr Polym. 2001;44(1):71–6.
124. Sánchez Á, Mengíbar M, Rivera-Rodríguez G, Moerchbacher B, Acosta N, Heras A. The effect of preparation processes on the physicochemical characteristics and antibacterial activity of chitooligosaccharides. Carbohydr Polym. 2017;157:251–7.
125. Laokuldilok T, Potivas T, Kanha N, Surawang S, Seesuriyachan P, Wangtueai S, et al. Physicochemical, antioxidant, and antimicrobial properties of chitooligosaccharides produced using three different enzyme treatments. Food Biosci. 2017;18:28–33.
126. Vishu Kumar AB, Varadaraj MC, Gowda LR, Tharanathan RN. Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of papain and Pronase, and their bactericidal action against Bacillus cereus and Escherichia coli. Biochemical Journal. 2005;391(2):167–75.
127. Shoaf K, Mulvey GL, Armstrong GD, Hutkins RW. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic Escherichia coli to tissue culture cells. Infect Immun. 2006;74(12):6920–8.
128. Xu X, Qiao Y, Peng Q, Gao L, Shi B. Inhibitory effects of YCW and MOS from Saccharomyces cerevisiae on Escherichia coli and Salmonella pullorum adhesion to Caco-2 cells. Front Biol (Beijing). 2017;12(5):370–5.
129. Bouckaert J, Mackenzie J, de Paz JL, Chipwaza B, Choudhury D, Zavialov A, et al. The affinity of the FimH fimbrial adhesin is receptor-driven and quasi-independent of Escherichia coli pathotypes. Mol Microbiol. 2006;61(6):1556–68.
130. Martinov J, Krstić M, Spasić S, Miletić S, Stefanović-Kojić J, Nikolić-Kokić A, et al. Apple pectin-derived oligosaccharides produce carbon dioxide radical anion in Fenton reaction and prevent growth of Escherichia coli and Staphylococcus aureus. Food Research International. 2017;100:132–6.
131. Rhoades J, Manderson K, Wells A, Hotchkiss Jr AT, Gibson GR, Formentin K, et al. Oligosaccharide-mediated inhibition of the adhesion of pathogenic Escherichia coli strains to human gut epithelial cells *in vitro*. J Food Prot. 2008;71(11):2272–7.

132. Li P jun, Xia J lan, Nie Z yuan, Shan Y. Pectic oligosaccharides hydrolyzed from orange peel by fungal multi-enzyme complexes and their prebiotic and antibacterial potentials. LWT-Food Science and Technology. 2016;69:203–10.
133. Li S, Li T, Zhu R, Wang N, Song Y, Wang S, et al. Antibacterial action of haw pectic oligosaccharides. Int J Food Prop. 2013;16(3):706–12.
134. Benhabiles MS, Salah R, Lounici H, Drouiche N, Goosen MFA, Mameri N. Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. Food Hydrocoll. 2012;29(1):48–56.
135. No HK, Park NY, Lee SH, Meyers SP. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. Int J Food Microbiol. 2002;74(1–2):65–72.
136. Zheng LY, Zhu JF. Study on antimicrobial activity of chitosan with different molecular weights. Carbohydr Polym. 2003;54(4):527–30.
137. Lee JH, Shim JS, Lee JS, Kim MK, Chung MS, Kim KH. Pectin-like acidic polysaccharide from Panax ginseng with selective antiadhesive activity against pathogenic bacteria. Carbohydrate Research. 2006;341(9):1154–63.
138. Wang Z, Yang Q, Wang X, Li R, Qiao H, Ma P, et al. Antibacterial activity of xanthan-oligosaccharide against Staphylococcus aureus via targeting biofilm and cell membrane. International Journal of Biological Macromolecules. 2020;153:539–44.
139. Piotrowski M, Wultańska D, Obuch-Woszczatyński P, Pituch H. Fructooligosaccharides and mannose affect Clostridium difficile adhesion and biofilm formation in a concentration-dependent manner. European Journal of Clinical Microbiology & Infectious Diseases. 2019;38(10):1975–84.
140. Di R, Vakkalanka MS, Onumpai C, Chau HK, White A, Rastall RA, et al. Pectic oligosaccharide structure-function relationships: Prebiotics, inhibitors of Escherichia coli O157: H7 adhesion and reduction of Shiga toxin cytotoxicity in HT29 cells. Food Chemistry. 2017;227:245–54.
141. Almagro-Moreno S, Pruss K, Taylor RK. Intestinal colonization dynamics of Vibrio cholerae. PLoS Pathog. 2015;11(5):e1004787.
142. Choi BK, Kim KY, Yoo YJ, Oh SJ, Choi JH, Kim CY. *In vitro* antimicrobial activity of a chitooligosaccharide mixture against Actinobacillus actinomycetemcomitans and *Streptococcus* mutans. Int J Antimicrob Agents. 2001;18(6):553–7.
143. Liu H, Du Y, Wang X, Sun L. Chitosan kills bacteria through cell membrane damage. Int J Food Microbiol. 2004;95(2):147–55.
144. Reffuveille F, Josse J, Vallé Q, Gangloff CM, Gangloff SC. Staphylococcus aureus Biofilms and their Impact on the Medical Field. The rise of virulence and antibiotic resistance in staphylococcus aureus. 2017;11:187.
145. Tin S, Lim CS, Sakharkar MK, Sakharkar KR. Synergistic combinations of chitosans and antibiotics in Staphylococcus aureus. Letters in Drug Design & Discovery. 2010;7(1):31–5.
146. Moon JS, Kim HK, Koo HC, Joo YS, Nam H mi, Park YH, et al. The antibacterial and immunostimulative effect of chitosan-oligosaccharides against infection by Staphylococcus aureus isolated from bovine mastitis. Appl Microbiol Biotechnol. 2007;75(5):989–98.
147. Fei Liu X, Lin Guan Y, Zhi Yang D, Li Z, de Yao K. Antibacterial action of chitosan and carboxymethylated chitosan. J Appl Polym Sci. 2001;79(7):1324–35.
148. Valcheva R, Dieleman LA. Prebiotics: Definition and protective mechanisms. Best Practice & Research Clinical Gastroenterology. 2016;30(1):27–37.
149. Servin AL. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol Rev. 2004;28(4):405–40.
150. Gopalakrishnan A, Clinthorne JF, Rondini EA, McCaskey SJ, Gurzell EA, Langohr IM, et al. Supplementation with galacto-oligosaccharides increases the percentage of NK cells and reduces colitis severity in Smad3-deficient mice. J Nutr. 2012;142(7):1336–42.
151. Ringel Y, Quigley EMM, Lin HC. Using probiotics in gastrointestinal disorders. The American Journal of Gastroenterology Supplements. 2012;1(1):34.
152. Matsuzaki T, Chin J. Modulating immune responses with probiotic bacteria. Immunol Cell Biol. 2000;78(1):67–73.

153. Cherbut C, Michel C, Lecannu G. The prebiotic characteristics of fructooligosaccharides are necessary for reduction of TNBS-induced colitis in rats. J Nutr. 2003;133(1):21–7.
154. He Y, Liu S, Kling DE, Leone S, Lawlor NT, Huang Y, et al. The human milk oligosaccharide 2′-fucosyllactose modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation. Gut. 2016;65(1):33–46.
155. Akbari P, Braber S, Alizadeh A, Verheijden KAT, Schoterman MHC, Kraneveld AD, et al. Galacto-oligosaccharides protect the intestinal barrier by maintaining the tight junction network and modulating the inflammatory responses after a challenge with the mycotoxin deoxynivalenol in human Caco-2 cell monolayers and B6C3F1 mice. The Journal of Nutrition. 2015;145(7):1604–13.
156. Cai Y, Folkerts J, Folkerts G, Maurer M, Braber S. Microbiota-dependent and-independent effects of dietary fibre on human health. Br J Pharmacol. 2020;177(6):1363–81.
157. Jeurink P v, van Esch BCAM, Rijnierse A, Garssen J, Knippels LMJ. Mechanisms underlying immune effects of dietary oligosaccharides. Am J Clin Nutr. 2013;98(2):572S-577S.
158. Kleessen B, Blaut M. Modulation of gut mucosal biofilms. British Journal of Nutrition. 2005;93(S1):S35–40.
159. Martin TA, Jiang WG. Loss of tight junction barrier function and its role in cancer metastasis. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2009;1788(4):872–91.
160. Cani PD, Possemiers S, van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009;58(8):1091–103.
161. Kleessen B, Hartmann L, Blaut M. Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. British Journal of Nutrition. 2003;89(5):597–606.
162. Plöger S, Stumpff F, Penner GB, Schulzke J, Gäbel G, Martens H, et al. Microbial butyrate and its role for barrier function in the gastrointestinal tract. Annals of the New York academy of sciences. 2012;1258(1):52–9.
163. Peng L, Li ZR, Green RS, Holzman IR, Lin J. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. J Nutr. 2009;139(9):1619–25.
164. Fachi JL, de Souza Felipe J, Pral LP, da Silva BK, Corrêa RO, de Andrade MCP, et al. Butyrate protects mice from Clostridium difficile-induced colitis through an HIF-1-dependent mechanism. Cell Rep. 2019;27(3):750–61.
165. Bilotta AJ, Cong Y. Gut microbiota metabolite regulation of host defenses at mucosal surfaces: implication in precision medicine. Precis Clin Med. 2019;2(2):110–9.
166. Paassen N, Vincent A, Puiman P, van der Sluis M, Witte-Bouma J, Boehm G, et al. The regulation of intestinal mucin MUC2 expression by short-chain fatty acids: implications for epithelial protection. Biochemical Journal. 2009;420:211–9.
167. Holscher HD, Davis SR, Tappenden KA. Human milk oligosaccharides influence maturation of human intestinal Caco-2Bbe and HT-29 cell lines. J Nutr. 2014;144(5):586–91.
168. Bhatia S, Prabhu PN, Benefiel AC, Miller MJ, Chow J, Davis SR, et al. Galacto-oligosaccharides may directly enhance intestinal barrier function through the modulation of goblet cells. Mol Nutr Food Res. 2015;59(3):566–73.
169. Asahara T, Shimizu K, Nomoto K, Hamabata T, Ozawa A, Takeda Y. Probiotic bifidobacteria protect mice from lethal infection with Shiga toxin-producing Escherichia coli O157: H7. Infect Immun. 2004;72(4):2240–7.
170. Chang EB, Leung PS. Intestinal water and electrolyte transport. In: The gastrointestinal system. Springer; 2014. p. 107–34.
171. Muanprasat C, Wongkrasant P, Satitsri S, Moonwiriyakit A, Pongkorpsakol P, Mattaveewong T, et al. Activation of AMPK by chitosan oligosaccharide in intestinal epithelial cells: Mechanism of action and potential applications in intestinal disorders. Biochem Pharmacol. 2015;96(3):225–36.
172. Sinclair HR, Smejkal CW, Glister C, Kemp F, van den Heuvel E, de Slegte J, et al. Sialyloligosaccharides inhibit cholera toxin binding to the GM1 receptor. Carbohydr Res. 2008;343(15):2589–94.

173. Idota T, Kawakami H, Murakami Y, Sugawara M. Inhibition of cholera toxin by human milk fractions and sialyllactose. Biosci Biotechnol Biochem. 1995;59(3):417–9.
174. Sinclair HR, de Slegte J, Gibson GR, Rastall RA. Galactooligosaccharides (GOS) inhibit Vibrio cholerae toxin binding to its GM1 receptor. Journal of Agricultural and Food Chemistry. 2009;57(8):3113–9.
175. Sethi A, Wands AM, Mettlen M, Krishnamurthy S, Wu H, Kohler JJ. Cell type and receptor identity regulate cholera toxin subunit B (CTB) internalization. Journal of the Royal Society Interface Focus. 2019;9(2):20180076.
176. Wands AM, Cervin J, Huang H, Zhang Y, Youn G, Brautigam CA, et al. Fucosylated molecules competitively interfere with cholera toxin binding to host cells. ACS Infect Dis. 2018;4(5):758–70.
177. El-Hawiet A, Kitova EN, Klassen JS. Recognition of human milk oligosaccharides by bacterial exotoxins. Glycobiology. 2015;25(8):845–54.
178. Pukin A v, Branderhorst HM, Sisu C, Weijers CAGM, Gilbert M, Liskamp RMJ, et al. Strong inhibition of cholera toxin by multivalent GM1 derivatives. ChemBioChem. 2007;8(13):1500–3.
179. Zomer-van Ommen DD, Pukin A v, Fu O, Quarles Van Ufford LHC, Janssens HM, Beekman JM, et al. Functional characterization of cholera toxin inhibitors using human intestinal organoids. Journal of Medicinal Chemistry. 2016;59(14):6968–72.
180. Haksar D, de Poel E, van Ufford LQ, Bhatia S, Haag R, Beekman J, et al. Strong inhibition of cholera toxin B subunit by affordable, polymer-based multivalent inhibitors. Bioconjug Chem. 2019;30(3):785–92.
181. Haksar D, van Ufford LQ, Pieters RJ. A hybrid polymer to target blood group dependence of cholera toxin. Organic & Biomolecular Chemistry. 2020;18(1):52–5.
182. Rabbani GH, Albert MJ, Rahman H, Chowdhury AK. Short-chain fatty acids inhibit fluid and electrolyte loss induced by cholera toxin in proximal colon of rabbit *in vivo*. Dig Dis Sci. 1999;44(8):1547–53.
183. El-Hawiet A, Kitova EN, Kitov PI, Eugenio L, Ng KKS, Mulvey GL, et al. Binding of Clostridium difficile toxins to human milk oligosaccharides. Glycobiology. 2011;21(9):1217–27.
184. Gerhard R. Receptors and binding structures for Clostridium difficile toxins A and B. Uptake and trafficking of protein toxins. 2016;79–96.
185. Nguyen TTH, Kim JW, Park JS, Hwang KH, Jang TS, Kim CH, et al. Identification of oligosaccharides in human milk bound onto the toxin A carbohydrate binding site of Clostridium difficile. Journal of Microbiology and Biotechnology. 2016;26(4):659–65.
186. Koleva PT, Valcheva RS, Sun X, Gänzle MG, Dieleman LA. Inulin and fructo-oligosaccharides have divergent effects on colitis and commensal microbiota in HLA-B27 transgenic rats. British Journal of Nutrition. 2012;108(9):1633–43.
187. May T, Mackie RI, Fahey GC, Cremin JC, Garleb KA. Effect of fiber source on short-chain fatty acid production and on the growth and toxin production by Clostridium difficile. Scand J Gastroenterol. 1994;29(10):916–22.
188. Veshnyakova A, Protze J, Rossa J, Blasig IE, Krause G, Piontek J. On the interaction of Clostridium perfringens enterotoxin with claudins. Toxins (Basel). 2010;2(6):1336–56.
189. Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM, Roy NC. Regulation of tight junction permeability by intestinal bacteria and dietary components. J Nutr. 2011;141(5):769–76.
190. Wrigley DM. Inhibition of Clostridium perfringens sporulation by Bacteroides fragilis and short-chain fatty acids. Anaerobe. 2004;10(5):295–300.
191. Newburg DS, Pickering LK, McCluer RH, Cleary TG. Fucosylated oligosaccharides of human milk protect suckling mice from heat-stabile enterotoxin of Escherichia coli. Journal of Infectious Diseases. 1990;162(5):1075–80.
192. Crane JK, Azar SS, Stam A, Newburg DS. Oligosaccharides from human milk block binding and activity of the Escherichia coli heat-stable enterotoxin (STa) in T84 intestinal cells. J Nutr. 1994;124(12):2358–64.
193. Anand S, Mandal S, Tomar SK. Effect of Lactobacillus rhamnosus NCDC 298 with FOS in Combination on Viability and Toxin Production of Enterotoxigenic Escherichia coli. Probiotics Antimicrob Proteins. 2019;11(1):23–9.

194. TAKASHI K, FLUITA I, KOBARI K. Effects of short chain fatty acids on the production of heat-labile enterotoxin from enterotoxigenic Escherichia coli. Jpn J Pharmacol. 1989;50(4):495–8.
195. Olano-Martin E, Williams MR, Gibson GR, Rastall RA. Pectins and pectic-oligosaccharides inhibit Escherichia coli O157: H7 Shiga toxin as directed towards the human colonic cell line HT29. FEMS Microbiol Lett. 2003;218(1):101–5.
196. Di R, Kyu E, Shete V, Saidasan H, Kahn PC, Tumer NE. Identification of amino acids critical for the cytotoxicity of Shiga toxin 1 and 2 in Saccharomyces cerevisiae. Toxicon. 2011;57(4):525–39.
197. Carey CM, Kostrzynska M, Ojha S, Thompson S. The effect of probiotics and organic acids on Shiga-toxin 2 gene expression in enterohemorrhagic Escherichia coli O157: H7. J Microbiol Methods. 2008;73(2):125–32.
198. Fukuda S, Toh H, Taylor TD, Ohno H, Hattori M. Acetate-producing bifidobacteria protect the host from enteropathogenic infection via carbohydrate transporters. Gut Microbes. 2012;3(5):449–54.
199. Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, et al. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 2011;469(7331):543–7.
200. Zumbrun SD, Melton-Celsa AR, Smith MA, Gilbreath JJ, Merrell DS, O'Brien AD. Dietary choice affects Shiga toxin-producing Escherichia coli (STEC) O157: H7 colonization and disease. Proceedings of the National Academy of Sciences. 2013;110(23):E2126–33.
201. Davie JR. Inhibition of histone deacetylase activity by butyrate. J Nutr. 2003;133(7):2485S-2493S.
202. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504(7480):446–50.
203. Nastasi C, Fredholm S, Willerslev-Olsen A, Hansen M, Bonefeld CM, Geisler C, et al. Butyrate and propionate inhibit antigen-specific CD8+ T cell activation by suppressing IL-12 production by antigen-presenting cells. Sci Rep. 2017;7(1):1–10.
204. Roberfroid M, Slavin J. Nondigestible oligosaccharides. Critical Reviews in Food Science and Nutrition. 2000;40(6):461–80.
205. Toro-Martín D, Arsenault BJ, Després JP, Vohl MC. Precision nutrition: a review of personalized nutritional approaches for the prevention and management of metabolic syndrome. Nutrients. 2017;9(8):913.

Chapter 6

Protective effects of alginate and chitosan oligosaccharides against *Clostridioides difficile* bacteria and toxin

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Abstract

Clostridioides difficile is the most dominant causative pathogen of nosocomial diarrhea, and *C. difficile* infection (CDI) is expected to become the most common healthcare-associated infection worldwide. Antibiotic-resistance mechanisms and disease recurrences lead to increased morbidity and mortality rates and therefore *C. difficile* infections carrying a high economic burden. *C. difficile*-induced pathogenicity is significantly attributed to its enterotoxin TcdA, which primarily targets Rho-GTPases involved in regulating cytoskeletal and tight junction (TJ) dynamics, thus leading to cytoskeleton breakdown, loss of cell-cell contacts, and ultimately to an increase of epithelial permeability. Hence, *C. difficile* and TcdA constitute attractive pharmacological targets. This study investigated whether two relatively recently emerged prebiotic fibers, alginate (AOS) and chitosan- (COS) oligosaccharides possess antipathogenic and barrier-protective properties against *C. difficile* bacteria and TcdA toxin, respectively. Based on our findings, COS, but not AOS, significantly reduced bacterial growth, though the minimum inhibitory concentration (MIC) was not reached. Cell cytotoxicity assays demonstrated that both oligosaccharides significantly attenuated TcdA-induced cell death. Challenging Caco-2 monolayers with increasing TcdA concentrations increased paracellular permeability in a concentration- and time-dependent trend as measured by TEER and LY flux assays, while none of the TcdA concentrations elicited IL-8 release. In this experimental setup, COS completely abolished, and AOS mitigated the deleterious effects of TcdA on the monolayer's integrity in a concentration dependent manner. Alterations in ZO-1 and occludin protein levels were not evident in any of the TcdA- or NDO-stimulated groups, nor in the control group. A calcium-switch assay revealed that both AOS and COS accelerate re-assembly of TJs, which could possibly contribute to enhanced barrier integrity during exposure to TcdA. Overall, this study established the antipathogenic and barrier-protective capacity of AOS and COS against *C. difficile* and its major toxin TcdA while revealing the ability of AOS and COS to promote TJ re-assembly in Caco-2 cells.

Keywords: *C. difficile*, TcdA, epithelial barrier, AOS, COS, TJs, TEER, LY, calcium-switch assay

1 Introduction

Clostridioides difficile is a Gram-positive, anaerobic, spore-forming, and toxin-producing bacillus. *C. difficile* is known as the most prevalent causative pathogen of nosocomial diarrhea, and *C. difficile* infection (CDI) has been estimated to cause 15%-25% of all cases of antibiotic-associated diarrhea (1,2). Strikingly, CDI is projected to become the most common healthcare-associated infection worldwide, even surpassing the methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (3,4). Once reaching the human intestine, this opportunistic bacterium can change into its vegetative disease-causing state. The colonic anaerobic environment and the presence of glycine and cholate derivatives facilitate its colonization and spores' germination. Under homeostatic conditions, the balanced gut microbiota populations prevent this by further processing the cholate derivatives (5). Nonetheless, upon induction of dysbiosis, principally due to broad-spectrum antibiotics consumption, bacterial germination and overgrowth are facilitated, while in parallel the disruption of intestinal flora grants *C. difficile* even more space in the intestine (5,6). CDI development is also associated with other risk factors, including advanced age, pregnancy, hypoalbuminemia, and impaired adaptive immunity (2,3,5). Clinical manifestations of CDI range from asymptomatic colonization, mild diarrhea, abdominal cramps, and fever to fulminant- and pseudomembranous- colitis, toxic megacolon, bowel perforation, sepsis, and ultimately death (2,4). The pathogenetic effects of *C. difficile* are majorly secondary to the activity of two exotoxins; toxin A (TcdA) and toxin B (TcdB), while certain strains produce the binary toxin *C. difficile* transferase (CDT). TcdA is a major virulence factor of *C. difficile*, and the causative agent of antibiotic-associated pseudomembranous colitis (7). TcdA is a large, single-unit, potent enterotoxin that acts via glycosylation of small GTP-binding proteins involved in the organization of cytoskeletal dynamics and is directly involved in the *C. difficile*-mediated inflammation and massive diarrhea (8). TcdA-mediated glycosylation of Rho (Rho, Rac, and Cdc42) proteins halts their interaction with downstream effectors and blocks Rho-dependent signaling pathways, thus causing both cytopathic and cytotoxic effects. Specifically, TcdA possesses glucosyltransferase activity, with a primary target the A-GTPase, a Ras homolog family member (RhoA-GTPases), which is a crucial regulator of actin cytoskeleton dynamics and tight junction assembly. TcdA inactivates the RhoA-GTPase by inducing its transition from the guanosine triphosphate (GTP)-bound form (active) to guanosine diphosphate (GDP)-bound form (inactive), ultimately leading to cytoskeletal breakdown and tight junction (TJ) disassembly (9) (**Figure 1**). Each TJ is formed by the assembly of various proteins (mainly ZO-1, -2, -3, occludin, claudins, and JAMs) and is located near the apical side of the lateral membrane of the epithelium. Disruption of TJs, the "gate-keepers" of the paracellular route and critical components of the epithelial barrier, results in impaired epithelial barrier integrity, known as the "leaky gut" condition. Increased intestinal permeability leads to acute colonic mucosa inflammation and excessive fluid loss, while the increased invasion of pathogens into the systemic circulation subsequently results in systemic inflammation and initiation of numerous diseases (10).

CDI not only causes high mortality but also carries an increased financial burden, especially since the development of antibiotic-resistance mechanisms and the disease recurrences (5,11). At present, available therapeutic strategies for CDI include the use of specific antibiotics against *C. difficile*, fecal transplantation, and surgery. However, severe and recurrent CDI treatment is still exigent, with limited treatment options available (9). Hence, both *C. difficile* and TcdA constitute appealing pharmacological targets; thus, identifying agents with antibacterial capacity against C. difficile or with barrier-protective properties against the TcdA-induced toxicity or even better with both abilities, is a very promising strategy. In this context, lately, there has been a growing interest in nutraceuticals, particularly in the prebiotic dietary fibers named non-digestible oligosaccharides (NDOs). NDOs possess multiple biological properties and confer innumerable health benefits via their ability to shape intestinal microbiota and the microbiota-related immune responses. Emerging evidence supports that NDOs also exert their health-beneficial effects microbiota-independently via direct interactions with intestinal epithelial and immune cells (12). Among the numerous NDO categories identified, alginate-oligosaccharides (AOS) and chitin/chitosan-oligosaccharides (COS) have drawn tremendous attention due to their versatile health-beneficial activities, including antimicrobial and intestinal barrier-reinforcing capabilities (13). AOS have been associated with prebiotic, antibacterial, immunoregulatory, anti-inflammatory, anti-apoptotic, neuroprotective, anti-tumor, anti-oxidative, and hypoglycemic activities, among others (14–16). These are acidic anionic carbohydrates obtained upon degradation or microbial fermentation of alginate, a biopolymer isolated from the cell walls of brown algae (14,17,18). AOS are low molecular weight (MW) linear polymers that contain 2-25 monomers and are composed of two types of two isomeric uronic acids: (1→4)-linked-α-L-guluronic acid (GulA/G) and (1→4)-linked-β-D-mannuronic acid (Man/M) (14) (**Supplementary figure 1A**). These monomers form either homooligomeric blocks (GG, G-blocks/MM, M-blocks) or hetero-oligomeric mixed sequences (G-M, GM-blocks) (18–20). The structural characteristics of AOS, i.e., spatial conformation, MW, G content (M/G ratio), and MG sequence, have a substantial impact on biological activity (16). Moreover, COS also exhibit a wide range of biological activities including prebiotic, anti-inflammatory, anti-tumor, antimicrobial, anti-oxidant, anti-HIV-1, anti-Alzheimer, and immunostimulatory abilities (21–24). COS are prepared from the deacetylation and degradation of chitin or depolymerization of chitosan (21,22,25). These are linear polymers consisting of β-(1→4)-linked N-acetyl-2-amino-2-deoxyglucose (N-acetyl-D-glucosamine/GlcNAc, acetylated unit A) and β-(1→ 4)-linked D-glucosamine (GlcN, deacetylated unit D) (21,24,26) (**Supplementary Figure 1B**). Chitin constitutes the second most abundant polymer in nature (22), found in the exoskeletons of crustaceans and arthropods and the cell walls of fungi, algae, and yeast with a high proportion of GlcNAc. By contrast, chitosan, which is mainly composed of GlcN, is rarer and can be extracted from the cell walls of specific fungi (18,21,27). COS are oligomers with an average MW<3.9 kDa (though not strictly), DP<20, and a high degree of deacetylation, DD>90% (DD: the molar units of GlcN in the COS backbone) (18,21,22,26). The chemical characteristics of COS mixtures, i.e., MW distribution (polydispersity or PD), DP, DD, and the sequence or pattern

of N-acetylation (PA)/charge distribution, have a tremendous impact on the exhibited physicochemical and ultimately biological properties (21,22).

Recently, accumulating evidence supports that both AOS and COS exert barrier-protective effects against various triggers known for inducing increased occurrence of epithelial permeability and leaky gut condition. Among them are inflammatory cytokines (i.e., TNF-α, INF-γ), lipopolysaccharide (LPS), dextran sulfate sodium (DSS), and enterotoxigenic *Escherichia coli (E. coli)*, all causing impairments of the epithelial monolayer (17,20,25,28,29). Both NDOs exert epithelial barrier protection via their prebiotic activity, i.e., by restoring imbalanced gut flora populations and abundance of their health-beneficial metabolites, short-chain fatty acids (SCFA) (19,30–34). Strikingly, apart from their microbiota-related barrier-protective activity, both NDOs have been reported to reinforce the intestinal barrier via direct interactions with intestinal epithelial cells, saving the integrity of TJ complexes responsible for maintaining a functional barrier (17,19,29,35–37). Here, the hypothesis that AOS and COS exert a protective role also in TcdA-induced epithelial injury was tested, using the Caco-2 cell line as a model for the human intestinal barrier. In addition, lately, there has been a great interest in NDOs as antimicrobial and anti-biofilm agents (38). AOS (38–40), but mostly COS (41–46) have been shown to exert various direct effects on enterotoxin-producing bacteria including *Escherichia coli*, *Streptococcus agalactiae*, and *S. aureus*, mediated by various mechanisms such as inhibition of bacterial growth, biofilm formation and adhesion mechanisms (47). Hence, in this study, the antigrowth capacity of AOS and COS against *C. difficile* was also examined, by performing a minimum inhibitory concentration (MIC) assay.

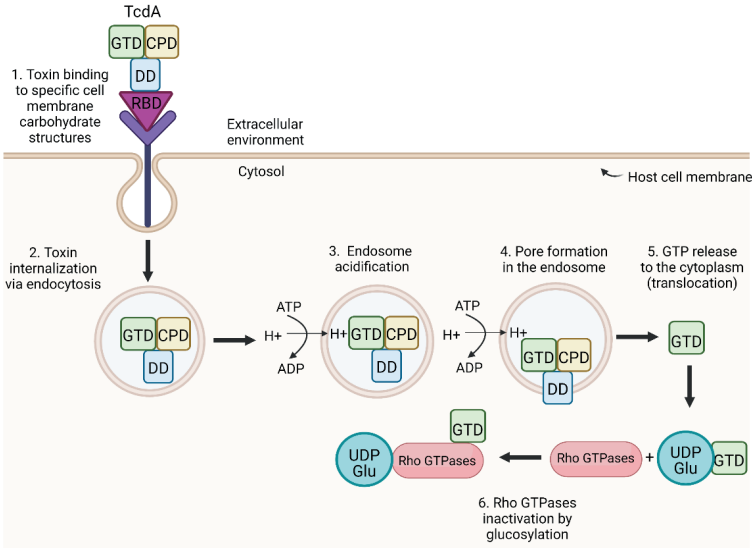

Figure 1. Mechanism of action mediating the TcdA-induced toxicity: (1) TcdA binding to specific host-cell surface carbohydrate structures via the B Domain (RBD) (2) receptor-mediated TcdA endocytosis (3) acidification-dependent translocation through the early endosomal membrane into the cytosol (4) pore formation via the hydrophobic region (D Domain, DD) (5) C Domain (CPD)-catalyzed toxin cleavage and release of A domain (GTD) from the endosome to the host cell cytosol (6) GTD-mediated transfer of glucose from UDP-glucose to Rho GTPases and inactivation (7) cytopathic (shrinking and rounding of cells, loss of cytoskeleton structure, disruption of cell-cell contacts, increased epithelial permeability), and cytotoxic effects (activation of inflammasome, increased ROS levels, induction of programmed cell death). RBD: receptor binding domain, DD: D Domain, CPD: cysteine protease domain, GTD: *N*-terminal glucosyltransferase domain.

2 Materials and methods

2.1 Bacterial strains and culture conditions

Clostridioides difficile bacteria (a kind gift from Len Lipman) were grown on BHIs plates (Biotrading, Mijdrecht, The Netherlands) for 24 h at 37°C, and thereafter, single colonies were inoculated in BHIs broth and incubated overnight under anaerobic conditions at 37°C. After incubation, bacterial growth was measured (OD600) and bacterial density was adjusted to OD600=1 and diluted 1/1, 1/10, 1/100, 1/1000 in BHIs to be used in MIC assays.

2.2 Cell culture

Colorectal adenocarcinoma (Caco-2) cells were purchased from the American Type Culture Collection (Code HTB-37) (Manassas, VA, USA, passages 27–45) and were cultured in Dulbecco's modified Eagle medium (DMEM, code 42430025), supplemented with 10% (v/v) inactivated fetal calf serum (FCS), 1% (v/v) L-glutamine, 1% (v/v) non-essential amino acids, penicillin (10,000 U/mL)/streptomycin (10,000 µg/mL). Cells were maintained in vented 75 cm^2 flasks in a humidified cell culture incubator with 5% CO_2 at 37 °C. Confluent cells (90%) were washed two times with phosphate-buffered saline (PBS) and detached

with 0.05% trypsin/0.54 mM ethylene diamine tetraacetic acid (EDTA). Caco-2 cells were seeded at a density of $3*10^4$/well in 96-well plates and were grown for 7 days (37 °C, 5% CO2) until a confluent monolayer was achieved. The medium was refreshed every other day.

2. 3 Oligosaccharides and TcdA

COS (purity > 90 %) and AOS (purity > 85 %) and were purchased from BZ Oligo Biotech Co., Ltd. (Qingdao, Shandong, China). COS originated from marine biological sources (shrimp and crab shells) and AOS was produced by the degradation of algin. The stock solutions of all NDOs were freshly prepared by dissolving them in BHIs (Minimum inhibitory concentration assay) or DMEM, before each experiment, and the pH of the solution was adjusted to 7.2–7.4 and finally the treatments were microfiltered using a syringe filter (0.2 µm, Corning, USA). *C. difficile* toxin A (TcdA) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and reconstituted in 250 µl sterile dH_2O to obtain the stock solution. For each experiment, the different concentrations of TcdA were freshly prepared by diluting the stock solution with DMEM.

2.4 Determination of the minimum inhibitory concentration (MIC)

The antibacterial capacity of AOS and COS against *C. difficile* was determined via analyzing the MIC following the method as described previously (39,48). NDOs were serially diluted in 96-well U-bottom polypropylene plates (Corning Costar, Cambridge, MA, USA), until reaching the final volume of 100 µL. Subsequently, 100 µL of bacterial inoculums (*C. difficile*) with OD_{600}=0.5 (approximately 10^8 colony-forming units [CFU]/mL) were added to the serially diluted NDOs (49,50). Thereafter, the plates were covered with sterile breathable film (VWR International, Amsterdam, Netherlands) and incubated overnight at 37°C under shaking conditions (160 rpm). Then, 100 µL of culture medium was transferred to 96-well F-bottom polystyrene microtiter plates (Corning Costar, Cambridge, MA, USA) and the signal was measured at 600 nm with a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany). Bacteria growth in BHIs without treatment served as positive control and BHIs alone was used as a negative control. Finally, the MIC was considered as the lowest concentration that inhibits bacterial growth by more than 90% in comparison to positive control groups.

2.5 Cell viability assays

Cell viability was examined using two different colorimetric assays. First, the 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) colorimetric assay (Sigma-Aldrich, St. Louis, Mo, USA) was performed to determine cell viability of the monolayers after stimulation with either AOS, COS, or TcdA. Briefly, Caco-2 cells were grown on 96 well-plates, and the confluent monolayers were exposed to different concentrations of NDOs (0.125%-4%) or TcdA (1 pg/mL-1 **µ**g/mL). After 48 and 24 h, for the NDOs and TcdA, respectively, MTT working solution (20 **µ**l, 5 mg/mL in PBS) was added to 100 µL of culture medium. Following 2 h of incubation, the supernatant was removed, cells were lysed with DMSO, and the absorbance was measured at 600 nm using iMark microplate

reader (BioRad). The viability rate of the Caco-2 cells was calculated based on the following equation: (mean absorbance of treatment cells/mean absorbance of control cells)*100.

Secondly, the lactate dehydrogenase (LDH) assay was conducted (Promega, Madison, WI USA). The release of LDH is a marker of cell membrane integrity; thus, measuring the levels of LDH in the culture supernatant is a stable index of the cellular injury extent. Caco-2 cells were grown on 96 well-plates, and upon reaching confluency, the monolayers were pre-treated with the NDOs (0.5%-2%) for 24 h. Then, the cells were triggered with either TcdA 10 ng/mL alone or in addition to AOS/COS for a further 24 h. The supernatant was collected after 3, 6, and 24 h, and LDH leakage into the supernatant was determined using a colorimetric reaction and reading of absorbance at 560 nm according to the manufacturer's protocol.

2.6 ELISA assay for CXCL8 secretion

Culture supernatants from the MTT assay for TcdA (1 pg/mL to 1 **µ**g/mL) were collected 24 hours post-exposure to quantify the inflammatory marker CXCL8 via an Enzyme-linked immunosorbent assay (ELISA). A corresponding CXCL8 kit (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions.

2.7 Transepithelial electrical resistance (TEER) measurement

Caco-2 cells were seeded on 0.33 cm^2 high pore density polyethylene terephthalate membrane Transwell® inserts with 0.4 µm pores (Corning Costar Corp., USA) placed in a 24-well plate, at a density of 0.1×10^5 cells/insert. The medium was refreshed every other day. Barrier integrity assays were started after obtaining a confluent monolayer at day 17–20 of culturing with TEER values in the range of ±700 $\Omega.cm^2$. To select one TcdA concentration for the subsequent experiments, cells were exposed apically to 100 ng/mL, 10 ng/mL, and 1 µg/mL TcdA and incubated for 24 h. Having determined TcdA 1 ng/mL as the optimal concentration, the cells were initially pre-treated with AOS/COS 0.5-2% for 24 h. Then, the cells were challenged with TcdA 1 ng/mL apically and a period of further 24 h of co-incubation followed. The integrity of the monolayer was determined prior to and after TcdA exposure (3, 6 and 24 h) by measuring TEER levels using a Millicell-ERS volt-ohmmeter (Millipore, Temecula, CA, USA). Average TEER values for untreated cell monolayers were in the range of 700 ± 20 $\Omega.cm^2$. The results are expressed as a percentage of the initial value.

2.8 Paracellular tracer flux assay

Paracellular permeability across the Caco-2 cell monolayer was determined by measuring the flux of the membrane-impermeable molecule lucifer yellow (LY). The transportation studies from the apical to the basolateral side were performed with 20 µg/mL of LY (molecular weight: 0.457 kDa, Sigma Chemical Co, St Louis, MO, USA), which was added to the apical compartment (200 µL) of the inserts, 24 hours post-TcdA exposure. Medium from the basolateral chamber was collected 5 hours after the tracer's addition. The amount of LY

in the basolateral compartment was determined by measuring the fluorescence intensity at excitation and emission wavelengths of 405 and 500-550 nm, respectively.

2.9 Calcium switch assay

Caco-2 cells grown on inserts were pre-treated with AOS/COS 0.5-2% added to the apical compartment for 24 hours and TEER values were measured. Subsequently, cells were washed with PBS and TJ protein complex was disrupted by incubation with 2 mM ethylene glycol-bis (2-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA, Sigma Chemical Co, St Louis, MO, USA) in calcium- and magnesium-free Hank's balanced salt solution (HBSS, Gibco, Invitrogen, CA, USA) for 20 min. Subsequently, the HBSS-EGTA was removed, cells were rinsed with PBS, and cell–cell contacts were allowed to re-establish by incubation with either complete cell culture DMEM (containing 2 mM $CaCl_2$) or in DMEM supplemented with the different AOS/COS concentrations. TEER values were measured during this recovery period to examine the TJ re-assembly progress every 2 h and for a time period of 10 h. The results are expressed as a percentage of initial value.

2.10 Western blot analysis

Caco-2 monolayers grown on inserts were pre-treated with or without AOS or COS for 24 h and exposed to TcdA for a further 24 h. Thereafter, cells were lysed using 50 µL RIPA lysis buffer/well (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany). Next, cells were washed with cold PBS, and cells were lysed with 50 µl RIPA lysis buffer containing protease inhibitors (Roche Applied Science, Penzberg, Germany). After 30 min incubation with RIPA buffer, cells were harvested and centrifuged at 16.000 *g* for 20 minutes to yield a clear lysate. The lysates were normalized for protein content and for total protein concentration assessment a BCA protein assay kit (Thermo Scientific) was used. Equal protein amounts of heat-denaturated nonreduced samples were separated by electrophoresis (Criterion™ Gel, 4–20% Tris–HCl, Bio-Rad Laboratories Inc.) and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad,Veenendaal,The Netherlands). Thereafter, membranes were blocked with PBS containing 0.05% (v/v) Tween-20 (PBST) and 5% (w/v) milk proteins for 2 h at room temperature. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies against ZO-1, occludin (1:1000, Invitrogen, Carlsbad, CA, USA) and monoclonal rabbit anti-human β-actin antibody (1:2000, Cell Signaling, Danvers, MA, USA) for equality of sample loading. Following washing with PBST, blots were incubated with appropriate horseradish peroxidase conjugated secondary antibodies (1:2000, Dako, Glostrup, Denmark) for 1 hour at room temperature. Next, membranes were washed in PBST and incubated in commercial ECL reagents (Amersham Biosciences, Roosendaal,The Netherlands) and finally exposed to X-ray film (Thermo Scientific, Antwerp, Belgium). The ChemiDoc™ MP imager (Bio-Rad Laboratories Inc.) was used to obtain the digital images, and signal intensities were quantified via the ImageJ 1.47 software and expressed as relative protein expression (optical density normalized with β-actin).

2.11 Statistical Analysis

Data were reported as mean values ± SEM of at least three independent experiments (n=3) routinely performed in triplicate (three wells/condition). Results were analyzed using Prism 8.0 GraphPad Software (GraphPad, San Diego, CA, USA). Statistical significance was determined using ANOVA followed by Bonferroni *post-hoc* test. Differences were considered statistically significant when $p < 0.05$.

3 Results

3.1 AOS and COS differentially affect *C. difficile* growth

To examine whether AOS and COS possess antigrowth activity against *C. difficile*, bacterial growth in BHIs in the absence or presence of ascending NDO concentrations was investigated. As shown in **Figure 2A**, the addition of AOS induced a significant decrease in the growth of *C. difficile*. This effect was evident already from the relatively low concentration of 0.5%, while the lowest concentration (0.25%) did not present any significant inhibitory capability. The maximal inhibitory growth (80%) was achieved by AOS 8%. Even though AOS 0.5-8% significantly reduced the growth of *C. difficile*, the minimum inhibitory concentration was not identified, since the maximum inhibition of growth did not reach 90%. Contrary to AOS, none of the COS concentrations (0.5-8%) affected the growth of *C. difficile* (**Figure 2B**).

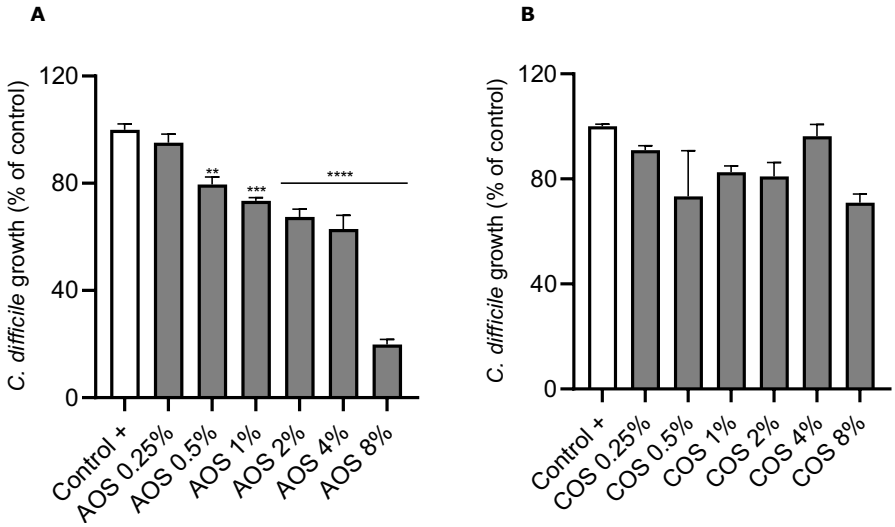

Figure 2. Effect of AOS and COS on bacterial growth of C. difficile: In order to examine the anti-growth capacity of the NDOs against *C. difficile*, a MIC assay was performed for AOS (A) and COS (B) using six 2-fold serial dilutions of each NDO. Control + represents the percentage of maximal bacterial growth without any treatment. Results are expressed as the percentage of the relative to the control-bacterial growth, as mean ± SEM of three independent experiments, each performed in triplicate. (**P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0 .0001: significantly different from the control +, as obtained using one-way ANOVA test).

3.2 NDOs and TcdA concentration selection

An MTT cell viability assay was conducted to select three concentrations per NDO for the subsequent experiments. After 48 hours of exposure, the highest concentration of both NDOs (4%) significantly decreased cell viability of Caco-2 cells (**Supplementary figure 2A, B**), thus the concentrations chosen for both AOS and COS were 0.5, 1, and 2%. Furthermore, the cells were triggered with 1 pg/mL to 1 µg/mL TcdA and incubated for 24 hours. The first three higher concentrations (10 ng/mL to 1 µg/mL) significantly reduced cell viability (**Supplementary figure 2C**). Three concentrations were selected for further experiments: 100 pg/mL and 1 ng/mL (non-cytotoxic), and 10 ng/mL (cytotoxic).

3.3 Exposure of the Caco-2 monolayer to TcdA fails to elicit IL-8 release

Cell culture supernatants from monolayers exposed to TcdA 1pg/mL to 1 µg/mL were collected 24 hours post-challenge to examine whether the toxin induces the release of CXCL8 from the Caco-2 cells. However, none of the TcdA concentrations resulted in release of CXCL8 (**Supplementary figure 3**).

3.4 Both NDOs decrease TcdA-induced cell cytotoxicity

Caco-2 monolayers were pre-treated with increasing AOS/COS concentrations and after 24 hours of incubation the cells were exposed to the cytotoxic concentration of 10 ng/mL, and co-incubated with either AOS or COS for further 3, 6, and 24 hours. AOS 1% and 2%, but not 0.5%, significantly decreased LDH leakage compared to untreated TcdA-challenged cells after 24 hours of co-incubation. By contrast, none of the AOS concentrations managed to alleviate TcdA-induced cytotoxicity after 3 and 6 hours (**Figure 3A**). COS 1% and 2% also exhibited cytoprotective activity as demonstrated by the significantly diminished LDH leakage already after 3 hours. This effect persisted after 6 and 24 hours of TcdA addition for COS 1% and 2%, respectively. COS 0.5% did not exert any significant protective effect, except the 6 hour post-TcdA challenge (**Figure 3B**).

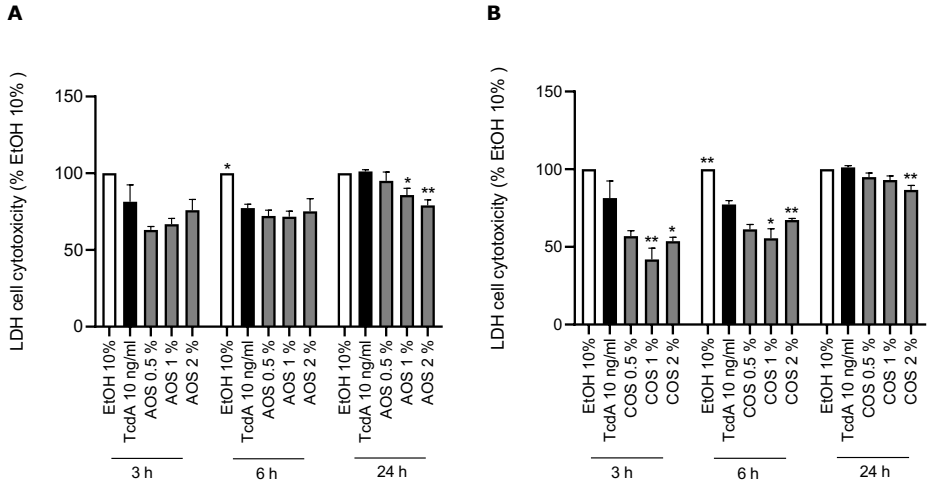

Figure 3. LDH cell cytotoxicity assay: Caco-2 monolayers were pre-treated with AOS or COS 0.5%-2% for 24 hours. Thereafter, cells were triggered with TcdA 10 ng/mL and a co-incubation period of further 3, 6, and 24 hours with either AOS (A) or COS (B) followed. TcdA-induced cell cytotoxicity was determined by measuring the LDH release to the cell culture supernatant. Ethanol 10% was used as positive control. Results are expressed as a percentage of positive control as mean ± SEM of three independent experiments, each performed in triplicate (*P ≤ 0.05, **P ≤ 0.01: significantly different from the TcdA-challenged cells, as obtained using one-way ANOVA test).

3.5 TcdA impairs Caco-2 monolayer's integrity

The deleterious effects of TcdA on the integrity of Caco-2 monolayers were investigated by stimulating the cells with increasing TcdA concentrations (100 pg/mL-10 ng/mL) for 24 hours and TEER was measured after 3, 6, and 24 hours post-challenge (**Supplementary figure 5A**). The highest concentration (10 ng/mL) significantly dropped TEER already after 3 hours, as well as at 6 and 24 hours post-challenge following a time-dependent trend. The non-cytotoxic concentrations 1 ng/mL and 100 pg/mL of TcdA, induced significant TEER reductions after 24 hours of toxin addition. Specifically, the effect of TcdA 1 ng/mL approached the levels of TcdA 10 ng/mL ($P<0.0001$), while the lowest concentration of 100 pg/mL was less potent ($P<0.05$) (**Figure 4A**). Similar results were obtained upon calculation of the total AUC, based on which the AUC for TcdA 1 ng/mL and 10 ng/mL were significantly lower compared to control (**Supplementary figure 5B**). To examine whether the TcdA-mediated disruption of TJs was reflected in an increased paracellular flux of impermeable tracers, the transportation of the cellular tracer LY from the apical to the basolateral chamber was determined. After 5 hours of LY addition to the apical compartment, both TcdA 1 ng/mL and 10 ng/mL resulted in a significant LY transport to the basolateral side, in accordance with the TEER results. By contrast, the lowest concentration did not affect the passage of LY (**Figure 4B**).

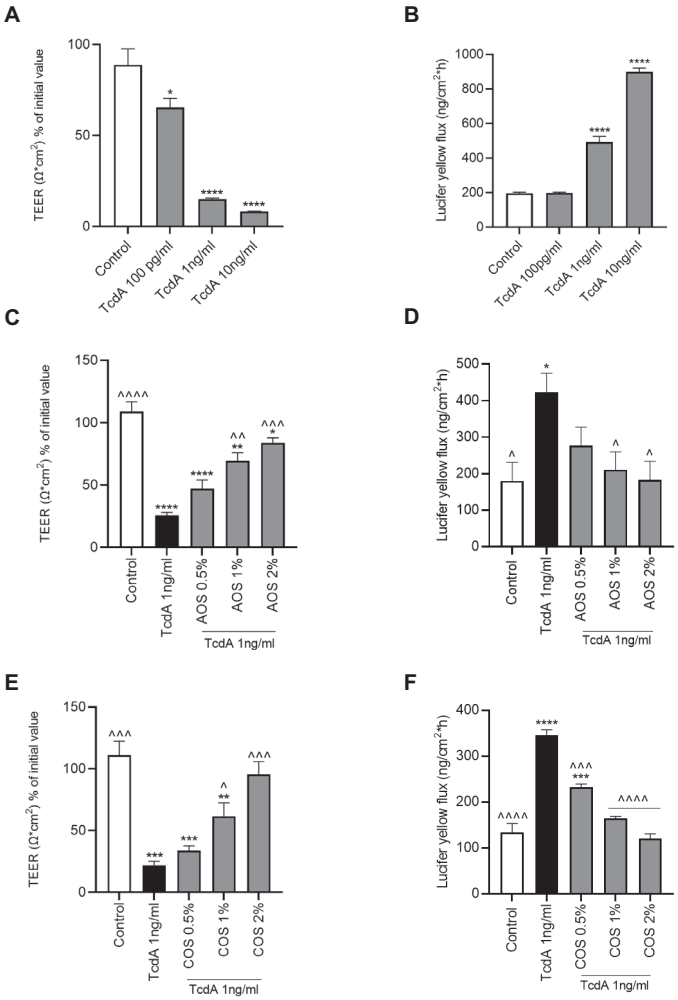

Figure 4. Effect of TcdA, AOS, and COS on epithelial barrier integrity: Caco-2 cells grown on Transwell® inserts for 17-21 days were (A, B) treated with increasing TcdA concentrations for 24 h or pre-treated with increasing (C, D) AOS or (E, F) COS concentrations for 24 h and subsequently triggered with TcdA 1 ng/mL, with a further TcdA/NDO co-incubation of 24 h. TEER values were measured prior to and after 24-hours TcdA challenge. For the paracellular tracer flux assay, LY (20 µg/mL) was added apically after 24 hours of TcdA exposure, and the amount of LY at the basolateral side was quantified after 5 hours. Results are expressed as a percentage of initial value (TEER) or the amount of tracer transported [ng/(cm2 × h)] as mean ± SEM of three independent experiments, each performed in triplicate (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001: significantly different from the unstimulated cells; ^P ≤ 0.05, ^^P ≤ 0.01, ^^^P ≤ 0.001, ^^^^P ≤ 0.0001: significantly different from the TcdA-stimulated cells, as obtained using one-way ANOVA test.

3.6 AOS and COS possess barrier-protective capability against TcdA-induced Caco-2 monolayer disruption

To evaluate the barrier-protective capacity of the NDOs against the noxious effects of TcdA on the epithelial monolayer, Caco-2 cells were pre-treated with AOS or COS (0.5-2%) for 24 hours. Then, TcdA 1 ng/mL was added apically and a co-incubation period of further 24 hours followed, and TEER measurements and LY flux assay were performed. As illustrated in **Figures 4C-F**, both NDOs prevented TcdA-mediated TEER decrease and increased LY transport in a concentration-dependent manner. Even though AOS 1% and 2% significantly alleviated the TEER reduction compared to TcdA-stimulated cells, TEER levels were also significantly lower from those of unstimulated cells. 2% AOS exerted the most potent effect, while the lowest concentration (0.5%) did not exert any protective effect (**Figure 4C**). Similarly, 1% and 2% AOS abolished the TcdA-induced increased flux of LY compared to TcdA-treated cells, while compared to control negative, pre-treatment with all of the AOS concentrations seemed to maintain the LY transportation at the levels of unstimulated cells (**Figure 4D**). As shown in **Figure 4E**, pre-treatment with COS 2% completely abolished the TcdA-induced TEER drop as resistance values were significantly different from TcdA-challenged monolayers. COS 1% also mitigated TcdA-induced TEER decreases, though presenting a significant difference compared to TcdA-challenged cells but also when compared to control. By contrast, COS 0.5% failed to prevent TEER decreases induced by TcdA. With regards to LY flux, all the COS concentrations were capable of significantly reducing LY transportation compared to TcdA-triggered monolayers, while compared to unstimulated cells, only COS 1% and 2% seemed to halt LY passage from the apical to the basolateral compartment. Finally, COS 0.5% failed to significantly attenuate increased tracer transport (**Figure 4F**). Then, the levels of ZO-1 and occludin were investigated via western blotting to examine whether the observed effects on paracellular permeability were accompanied by alterations on TJ protein levels. Interestingly, TcdA 1 ng/mL did not induce any changes in any of these TJ proteins, and no effect of treatment with AOS or COS could be observed (**Supplementary figure 6**).

3.7 Both NDOs facilitate the re-assembly of TJs after calcium deprivation in Caco-2 cells

To examine whether AOS and COS can accelerate the re-assembly of intercellular junction complexes under dynamic disassembly/re-assembly conditions, a calcium switch assay was conducted. In the presence of 2% AOS, re-establishment of cell-cell contacts already occurred 2 hours after calcium recovery (P:0.0547), a significant difference from the unstimulated cells also after 4 and 8 hours. AOS 1% also enhanced the TJ re-assembly during 4 and 8 hours, while AOS 0.5% did not exert any significant effect compared to control (**Figure 5A**). In addition, the corresponding total area under the curve (AUC) for AOS 2% was significantly different compared to control, contrary to the lowest concentrations (**Supplementary figure 7A**). COS 2% stimulated the re-assembly of TJs after 2, 6 (P:0.0537), and 10 hours of calcium replenishment. The lower concentrations (1%, 0.5%) did not induce any significant effect on TEER restoration (**Figure 5B**). Finally,

calculation of the total AUC indicated that only the effect of 2% COS showed a trend toward significance (P:0.0575) **(Supplementary figure 7B**).

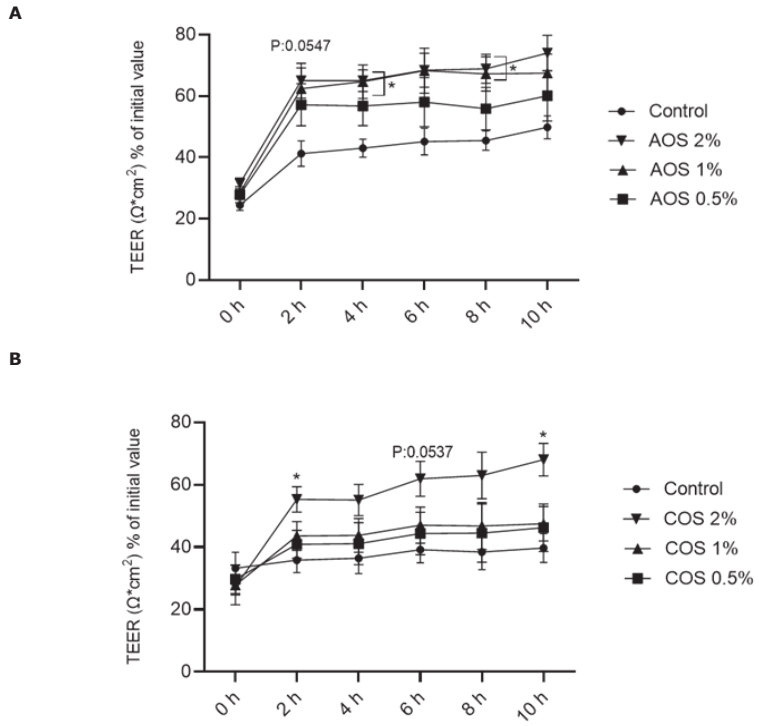

Figure 5. Calcium switch assay for AOS and COS: In order to examine whether the NDOs accelerate TJ re-assembly, Caco-2 cells grown on Transwell® inserts were pre-treated with increasing AOS/COS concentrations (24 h) prior to transient calcium deprivation with HBSS-EGTA to disrupt intercellular junctions. TEER values were measured during recovery (0, 2, 4, 6, 8, 10 h) in complete, calcium containing DMEM supplemented with either AOS (A) or COS (B). Results are expressed as a percentage of initial value as mean ± SEM of three independent experiments, each performed in triplicate (*P ≤ 0.05: significantly different from the unstimulated cells, as obtained using one-way ANOVA test for each time point separately).

4 Discussion

CDI is one of the major causes of nosocomial infection, which initially mainly occurred in developed countries, but is now emerging as a global threat. Development of resistance to antibiotics and the emergence of hypervirulent strains are considered as drivers of CDI outbreaks with significantly high mortality rates (6,51). CDI is caused by the opportunistic bacterium *C. difficile,* whose pathogenetic effects are mainly caused by its exotoxins. TcdA is a highly potent enterotoxin which is the major cause of *C. difficile* enterotoxicity via the induction of both cytopathic and cytotoxic effects and is a well-established disruptor of epithelial TJs (52). TJs of the intestinal epithelium constitute a key element

of the intestinal barrier as these intercellular protein complexes seal the paracellular space between neighboring cells and form a continuous intercellular barrier across the interspace of epithelial cells. Impaired TJ functionality, followed by permeability to luminal contents, induces inflammatory and immune responses, ultimately triggering the leaky gut syndrome. The primary target of TcdA is the inactivation of RhoA, a crucial regulator of actin cytoskeleton and TJ assembly. Inactivation of this small GTPase by glycosylation results in an alteration of cellular structure and impaired TJ integrity. Subsequently, the increased epithelial permeability triggers acute inflammatory and immune responses that further contribute to the leaky gut and excessive secretion of ions. NDOs are prebiotics that are able to selectively stimulate the growth of health-promoting bacteria and, via their fermentation products, exert numerous beneficial effects for the host, including preservation of a well-functioning mucosal barrier and homeostatic immune system. Apart from their microbiota-related beneficial activity, emerging evidence demonstrates that numerous NDOs also have direct barrier-protective properties, including direct induction of TJ signaling leading to protection from various TJ-disrupting triggers such as pathogens, toxins, inflammation-inducing cytokines, and LPS (12). Moreover, numerous NDO categories have been shown to exert antibacterial activity via inhibition of bacterial growth, adhesion, biofilm formation, among others (18,27). Among these oligosaccharides, AOS and COS seem very promising due to their versatile properties, though their direct antimicrobial and barrier-reinforcing abilities have not been widely investigated. Here, we addressed the hypothesis that AOS and COS can affect the growth of *C. difficile* and exert direct barrier protection against TcdA-mediated disruption of the epithelial layer.

Initially, the potential antibacterial activity of AOS and COS against *C. difficile* was investigated. Previous studies have revealed that both NDOs reduce the growth of other enterotoxin-producing bacteria *in vitro*. AOS significantly decreased the growth of *Streptococcus agalactiae* (38) and *E. coli* (39). COS inhibited the growth of *E. coli* (43,44,46), *Bacillus cereus* (46,53,54), and *S. aureus* (43,54,55). Here, based on the MIC assay results, AOS significantly decreased the bacterial growth already from the low concentration of 0.25%, while the best effect was exhibited by the highest concentration of 8%. However, despite significantly reducing bacterial growth by 80%, this cannot be characterized as inhibitory concentration, as the MIC is considered as the lowest concentration that inhibits bacterial growth by more than 90% compared to positive control groups. On the other hand, COS did not have an effect on *C. difficile* growth with a maximal reduction of 29% induced by COS 8%. This is the first time that the possible antibacterial effects of AOS and COS are examined against *C. difficile*. A recent study demonstrated that FOS, another well-established group of prebiotic oligosaccharides, exerted antibacterial effects against *C. difficile* via the inhibition of biofilm formation and cell adhesion (56). The differential antibacterial activity exerted by all these three NDOs could be explained by their differences with respect to their physicochemical characteristics. FOS are composed of D-fructose monomers usually with β-(2→1) linkages and a terminal α-(1→2) linked D-glucose residue and are uncharged in normal pH. The main components of AOS are 1,4-

linked β-D-mannuronic acid and 1,4-linked α-L-guluronic acid and of COS 1,4-linked GlcNAc and 1,4-linked GlcN, and carry negative and positive charges, respectively (18). As a result, even though these NDOs share similarities due to their carbohydrate nature, they have quite different physicochemical characteristics, affecting eventually their interactions with surface receptors or other types of molecule-molecule interactions. Considering the multiple antimicrobial effects of both AOS and COS against numerous Gram + and Gram – bacteria, including motility and QS-signaling inhibition, and pathogenic membrane disruption, further studies regarding the anti-pathogenic effects of AOS and COS against *C. difficile*, and the investigation of structure-function relationships, seem very promising.

TcdA mediates cell cytotoxicity by inducing inflammatory responses, oxidative stress, and programmed cell death. Previous studies on IECs have demonstrated that both NDOs have anti-apoptotic potential *in vitro*. AOS reduced TNF-α-induced apoptotic rates in IPEC-2 monolayers via inhibition of the cell surface death receptor TNFR1-initiated caspase-8-mediated pathway, thus halting the extrinsic apoptotic pathway (17). In another study AOS inhibited LPS binding to TLR-4 receptors of IPEC-2 cells and suppressed TLR-4/NF-κB-mediated apoptosis (19). Similarly, COS prevented LPS binding to T84 cells and reduced TNF-α- and oxidative stress-induced apoptosis (25). In this study we sought to investigate the possible protective abilities of AOS and COS against the TcdA-induced cytotoxic and cytopathic effects. Based on the LDH release assay, pre-treatment with AOS (1%, 2%) or COS (2 %) mitigated the Caco-2 cell cytotoxicity caused by 24 h incubation with 10 ng/mL TcdA (cytotoxic concentration). In addition, COS 1%, 2%, and COS 0.5, 1% exhibited a cytoprotective effect already after 3 and 6 h respectively, though the exact mechanisms underlying these anti-apoptotic effects were not further investigated. Interestingly, ELISA results showed that none of the toxin concentrations used (TcdA 1 pg/mL to 1 μg/mL) managed to elicit IL-8 release. This is in accordance with previous studies demonstrating the inability of TcdA in this concentration range on causing IL-8 production by Caco-2 monolayers (57,58). By contrast, in other studies using HT-29 and T84 monolayers, TcdA mediated the release of IL-8 (58,59), while in a study on Caco-2 cells, TcdA managed to induce TNF-α and IL-6 release (9). Even though TcdA is a well-recognized inflammatory enterotoxin, little is known regarding the underlying mechanisms of innate immune system activation and stimulation of pro-inflammatory cytokine release (60). Further studies are necessary to elucidate under which conditions TcdA elicits inflammatory response, and to determine the implicated cellular pathways.

Next, Caco-2 monolayers were grown on Transwell® inserts to investigate the use of AOS and COS in providing protection against TcdA-mediated disruption of epithelial barrier function. The integrity of the monolayer was assessed by measurement of TEER and apical to basolateral flux of LY. TEER measurements are a standard technical approach to evaluate barrier properties and dynamics *in vitro* and indicate the ionic conductance of the paracellular pathway (61). Transport studies using nonelectrolyte tracers indicate the TJ pore size and the paracellular water flow (62). Numerous studies have demonstrated the

TcdA-induced cytopathic effects, principally involving disruption of intercellular cell-cell junctions, and thus increased epithelial permeability *in vitro* (9,51,52,63). Exposure of the Caco-2 monolayers to TcdA 100 pg/mL, 1 ng/mL (non-toxic concentrations 24 h post-exposure) and 10 ng/mL (toxic concentration 24 h post-exposure) impaired the monolayer's integrity time- and concentration- dependently. TcdA 10 ng/mL induced a significant TEER drop already at the 3rd hour of incubation, in accordance with the LDH results which demonstrated that the TcdA-induced cytotoxicity at this concentration is evident already 3 hours post-exposure. On the other hand, 100 pg/mL, and 1 ng/mL TcdA significantly decrease TEER only after an incubation period of 24 hours. Moreover, in agreement with the TEER results, challenge with 1 and 10 ng/mL TcdA for 24 hours, resulted in a significant transportation of LY from the apical to the basolateral compartment, while LY flux for TcdA 100 pg/mL was comparable with levels of unstimulated cells. Hence, considering that 100 pg/mL and 1 ng/mL TcdA do not induce cell death when added to Caco-2 monolayers for 24 hours, while 10 ng/mL TcdA does, we can claim that TcdA 100 pg/mL and especially TcdA 1 ng/mL exerted cytopathic effects which led to increased paracellular permeability, while the acute TEER drop induced by TcdA 10 ng/mL was due to its cytotoxicity.

Here, we sought to investigate the protective potential of AOS and COS against the TcdA-mediated cytopathic effects, which are reflected by an increased paracellular permeability. To examine the protective abilities of these NDOs regarding the monolayer's integrity, the selection of a non-cytotoxic TcdA concentration was required. Having established 1 ng/mL TcdA as a cytopathic concentration based on both TEER and LY transport assays, the barrier-protective potential of AOS and COS was examined. Both NDOs have been previously shown to confer barrier protection *in vitro* against various stimuli known for disrupting epithelial TJs. AOS reinforced the epithelial integrity of IPEC/J2 monolayers in a mannose receptor (MR)-dependent manner (35,64) and saved the monolayer's integrity from TNFα (17), LPS (19) challenges by alleviating inflammation. COS have also been shown to promote TJ integrity via an 5' AMP-activated protein kinase (AMPK)-mediated acceleration of TJ re-assembly in T84 monolayers (36). Moreover, COS prevented barrier impairments of LPS-challenged T84 and IPEC-J2 cells (25,37), of TNF-a-challenged IPEC-J2 cells (65), and of DSS-challenged Caco-2 cells (29), by suppressing inflammatory responses. In this study, both AOS and COS succeeded in protecting the integrity of the monolayer in a concentration-dependent manner. The highest COS and AOS concentrations (1-2%) abrogated and alleviated, respectively, the TcdA-induced TEER drop. Both NDOs (1-2%) completely prevented the flux of LY from the apical to the basolateral chamber. To investigate whether changes in TJ protein levels accompany the observed effects, a western blot analysis was performed. Strikingly, despite the evident TcdA-mediated increased paracellular permeability, no alterations at the mRNA expression of two crucial TJ proteins, ZO-1 and occludin, were observed. In previous studies using Caco-2 cells, TcdA challenge decreased ZO-1 and occludin mRNA expression and abundance, though in all of these studies, the TcdA concentration applied was cytotoxic (9,52,66). TcdA has a time- and concentration-dependent toxicity thus, a plausible explanation for the

absence of TJ protein level changes is that the concentration of 1 ng/mL was not capable of suppressing TJ protein expression within this time frame. Thus, it is possible that the TcdA concentration used here, even though not cytotoxic, managed to impair the integrity of the Caco-2 monolayers via mislocalization of TJ proteins, but without altering their expression, as an early cellular response to TcdA. Furthermore, members of the claudin family of tight junction molecules might also be associated with the observed impact of TcdA (67), however, additional research is required to confirm this connection. Previous studies have demonstrated that the TcdA-mediated increased paracellular permeability is not necessarily the aftermath of actin cytoskeleton depolymerization and that the early increases in TJ permeability are mediated by signaling events, including activation of protein kinase C isoforms α/β (PKCα/PKCβ) (68,69). The PKC family of isozymes is involved in the regulation of TJs via direct phosphorylation of TJ proteins including ZOs and occludin, and the conventional PKCs (α, β1, β2, and γ) have been associated with TJ disassembly, TEER decreases, and TJ mislocalization (70). Moreover, in a previous study using T84 monolayers, TcdA impaired the monolayer's integrity as indicated by TEER and tracer flux assays, though without affecting ZO-1 and occludin protein levels, in agreement with our results. Confocal microscopy results showed that TcdA induced displacement of TJ proteins (ZO-1, -2, occludin) from the lateral membrane of TJs and F-actin disorganization. These effects were caused by the TcdA-mediated decreased association of the cytoplasmic plaque protein ZO-1 with the actin cytoskeleton, and this event was accompanied by a decreased pool of occludin and its internalization from the lateral TJ membrane since the functions of this TJ protein require binding to ZO proteins. Hence, TcdA-mediated increase of paracellular permeability was caused by the disassembly of TJs rather than due to changes in TJ proteins abundance (71,72), which could also be the case also for our study.

To explore the barrier-reinforcing properties of AOS and COS, a calcium switch assay was performed. This assay is based on the principle that intercellular TJs are disrupted upon deprivation of extracellular Ca^{2+}, leading to a marked TEER decrease. Once Ca^{2+} is replenished, TJs are re-assembled to the membrane periphery close to the apical surface (73). As mentioned above, COS have been previously shown to accelerate TJ re-assembly in T84 monolayers via a CaSR-Gq-PLC-IP3-CaMKKβ-dependent pathway that leads to AMPK stimulation (36). AMPK stimulation does not seem to be cell-line specific, as COS resulted to increased pAMPK levels also in Caco-2 and HT-29 monolayers. Notably, FOS also promoted TJ dynamics via induction of intracellular Ca^{2+} signaling and acceleration of TJ re-assembly, following the exact mechanism as COS in T84 monolayers (74). Here, pre-treatment of Caco-2 monolayers with 2% COS promoted the re-assembly of the TJ complex, with significant differences from the control group already after 2 hours of incubation, which also persisted after 6 and 10 hours. Moreover, AOS also presented this potential since 1% and 2% AOS significantly increased TEER compared to unstimulated cells at various time points, though the highest concentration was more effective. Based on a study using Caco-2 cells, the TJ re-assembly process depends on the activation of AMPK, a crucial TJ assembly/disassembly regulator, and that AMPK stimulation ensures a better recovery of epithelial

barrier function after injury (75). Thus, like FOS and COS, AOS seem to stimulate AMPK signaling via interactions with a Ca^{2+} sensing receptor (CaSR) of the epithelial cell surface. Considering the importance of AMPK in the facilitation of TJ recovery, we speculate that the barrier-protective effects against TcdA-induced epithelial damage are, at least in part, mediated by stimulation of the AMPK signaling. Furthermore, it is well-established that the receptor binding domain (B Domain) of TcdA interacts with carbohydrate structures containing galactose (Gal)- and GlcNAc found in the epithelial cell surface (5). Previous studies demonstrated that such a receptor is the trisaccharide Gal-α-(1,3)-Gal-β-(1,4)-GlcNAc present in the intestine of infant hamsters, but not expressed in humans (76,77). Nonetheless, it has been revealed that TcdA binds to carbohydrate antigens designated X, I, and Y which are existent on the intestinal epithelium of humans and have a type 2 core Gal-β-(1,4)-GlcNAc, with the NAc part being crucial for toxin binding. However, the single TcdA binding site has affinity for multiple carbohydrate ligands (78). Hence, another plausible mechanism underlying the AOS and COS-mediated protection against TcdA toxicity, may involve antagonism for the glycoconjugate receptors of the host epithelial cell membranes targeted by TcdA. Finally, taking into account that the main components of COS are 1,4-linked GlcNAc and 1,4-linked GlcN, it is highly likely that the superiority of this NDO over AOS regarding the protection against TcdA toxicity, is directly linked to the presence of the GlcNAc monomers.

5 Conclusion

The objective of this study was to evaluate the protective effects of AOS and COS against a major causative pathogen of hospital-acquired infection, the bacterium *C. difficile,* as well as against the deleterious effects of its principal enterotoxin, TcdA. Based on our findings, AOS managed to significantly reduce bacterial growth following a concentration-dependent trend, though the minimum inhibitory concentration was not identified. By contrast, treatment of *C. difficile* with COS failed to significantly affect bacterial growth. Nevertheless, both NDOs appeared to be quite successful regarding the TcdA-mediated cytopathic and cytotoxic effects. Both AOS and COS significantly decreased TcdA-induced cytotoxicity of Caco-2 monolayers, with the latter presenting a stronger cytoprotective capability. Next, based on the barrier integrity assays conducted, both NDOs protected the epithelial barrier from TcdA-mediated cell-cell junctions functionality disruption, though COS presented a more robust barrier-protective profile. Another interesting finding was that both NDOs accelerated the re-assembly of epithelial TJs of Caco-2 cells and thus can strengthen the intestinal barrier via promoting the sealing of the paracellular route. This AMPK-mediated barrier reinforcement is possibly, at least partly, related to the protective effects against TcdA. Considering the carbohydrate nature of AOS and COS with the cell surface binding structures of TcdA, receptor antagonism is another plausible explanation for the observed effects. In **Supplementary figure 8**, a synopsis of the concluding remarks as obtained by this study can be found. Finally, these findings uncover two very promising agents, AOS and COS, against *C. difficile* bacteria and toxin, opening the route for further

studies into alternative therapeutic strategies against CDI, while in parallel revealing the cytoprotective and barrier-enhancing abilities of these NDOs. Further studies are warranted, though, to unravel the underlying mechanisms by which AOS and COS exert their health-beneficial effects.

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Author Contributions

Conceptualization MA, MM, GF, RP, KS and SB; methodology, MA, MM; software, MA, MM; validation, MA, MM and SB; formal analysis, MA, MM; investigation, MA, MM; resources, KS, GF; data curation, MA, MM; writing—original draft preparation, MM, MA; writing—review and editing, KS, SB, GF and RP; visualization, MA, MM; supervision, GF, SB; project administration, GF, RP, KS, SB; funding acquisition, GF, SB and RP. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

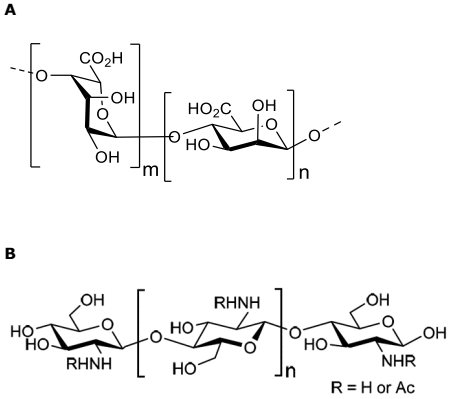
1. Chandrasekaran R, Lacy DB. The role of toxins in clostridium difficile infection. FEMS Microbiol Rev. 2017;41(6):723–50.
2. Bartlett JG, Gerding DN. Clinical recognition and diagnosis of Clostridium difficile infection. Clin Infect Dis. 2008;46(SUPPL. 1).
3. Alvarez AM, Rathore MH. Clostridium difficile Infection in Children. Adv Pediatr. 2019;66:263–80.
4. Banawas SS. Clostridium difficile Infections: A Global Overview of Drug Sensitivity and Resistance Mechanisms. Biomed Res Int. 2018;2018.
5. Bella S Di, Ascenzi P, Siarakas S, Petrosillo N, Masi A. Clostridium difficile Toxins A and B : Insights into Pathogenic Properties and Extraintestinal Effects. 2016;1–25.
6. Alyousef AA. Clostridium difficile: Epidemiology, pathogenicity, and an update on the limitations of and challenges in its diagnosis. J AOAC Int. 2018;101(4):1119–26.
7. Just I, Selzer J, Von Eichel-Streiber C, Aktories K. The low molecular mass GTP-binding protein Rho is affected by toxin A from Clostridium difficile. J Clin Invest. 1995;95(3):1026–31.
8. Skinner AM, Phillips ST, Merrigan MM, O'Leary KJ, Sambol SP, Siddiqui F, et al. The Relative Role of Toxins A and B in the Virulence of Clotridioides difficile. J Clin Med. 2020;10(1):96.
9. Pietrangeli P, Corpetti C, Seguella L, Del Re A, Pesce M, Vincenzi M, et al. Lathyrus sativus diamine oxidase reduces Clostridium difficile toxin A-induced toxicity in Caco-2 cells by rescuing RhoA-GTPase and inhibiting pp38-MAPK/NF-κB/HIF-1α activation. Phyther Res. 2021;35(1):415–23.
10. Camilleri M, Enteric C, Translational N, Clinic M. The Leaky Gut: Mechanisms, Measurement and Clinical Implications in Humans. 2020;68(8):1516–26.
11. Voth DE, Ballard JD. Clostridium difficile toxins: Mechanism of action and role in disease. Vol. 18, Clinical Microbiology Reviews. American Society for Microbiology (ASM); 2005. p. 247–63.
12. Cai Y, Folkerts J, Folkerts G, Maurer M, Braber S. Microbiota-dependent and -independent effects of dietary fibre on human health. Br J Pharmacol. 2020;177(6):1363–81.
13. Palaniappan A, Antony U, Emmambux MN. Current status of xylooligosaccharides: Production, characterization, health benefits and food application. Trends Food Sci Technol. 2021;111(February):506–19.
14. Mrudulakumari Vasudevan U, Lee OK, Lee EY. Alginate derived functional oligosaccharides: Recent developments, barriers, and future outlooks. Carbohydr Polym. 2021;267(May):118158.
15. Liu J, Yang S, Li X, Yan Q, Reaney MJT, Jiang Z. Alginate Oligosaccharides: Production, Biological Activities, and Potential Applications. Compr Rev Food Sci Food Saf. 2019 Nov;18(6):1859–81.
16. Xing M, Qi C, Yu W, Han X, Jiarui Z, Qing Z, et al. Advances in research on the bioactivity of alginate oligosaccharides. Mar Drugs. 2020;18(3).
17. Wan J, Zhang J, Yin H, Chen D, Yu B, He J. Ameliorative effects of alginate oligosaccharide on tumour necrosis factor-α-induced intestinal epithelial cell injury. Int Immunopharmacol [Internet]. 2020;89(August):107084. Available from: https://doi.org/10.1016/j.intimp.2020.107084
18. Asadpoor M, Ithakisiou G.N PAJH, Pieters R, Folkerts G, Braber and S. Non-digestible oligosaccharides and short chain fatty acids and their toxins. Toxins (Basel). 2021;13(3):175.
19. Wan J, Zhang J, Xu Q, Yin H, Chen D, Yu B, et al. Alginate oligosaccharide protects against enterotoxigenic Escherichia coli-induced porcine intestinal barrier injury. Carbohydr Polym. 2021;270(March).
20. Wan J, Zhang J, Chen D, Yu B, Mao X, Zheng P, et al. Alginate oligosaccharide alleviates enterotoxigenic: Escherichia coli-induced intestinal mucosal disruption in weaned pigs. Food Funct. 2018;9(12):6401–13.
21. Muanprasat C, Chatsudthipong V. Chitosan oligosaccharide: Biological activities and potential therapeutic applications. Pharmacol Ther. 2017;170:80–97.
22. Naveed M, Phil L, Sohail M, Hasnat M, Baig MMFA, Ihsan AU, et al. Chitosan oligosaccharide (COS): An overview. Int J Biol Macromol. 2019;129:827–43.
23. Kumar A, Kumaar A. The virtuous potential of chitosan oligosaccharide for promising biomedical applications. J Mater Res. 2020;35(9):1123–34.

24. Lopez-Santamarina A, Mondragon A del C, Lamas A, Miranda JM, Franco CM, Cepeda A. Animal-origin prebiotics based on chitin: An alternative for the future? a critical review. Foods. 2020;9(6):1–20.
25. Yousef M, Pichyangkura R, Soodvilai S, Chatsudthipong V, Muanprasat C. Chitosan oligosaccharide as potential therapy of inflammatory bowel disease: Therapeutic efficacy and possible mechanisms of action. Pharmacol Res [Internet]. 2012;66(1):66–79. Available from: http://dx.doi.org/10.1016/j.phrs.2012.03.013
26. Guan G, Abul Kalam Azad M, Lin Y, Kim SW, Tian Y, Liu G, et al. Biological effects and applications of chitosan and chito-oligosaccharides. Front Physiol. 2019;10(MAY):1–10.
27. Asadpoor M, Peeters C, Henricks PAJ, Varasteh S, Pieters RJ, Folkerts G, et al. Anti-pathogenic functions of non-digestible oligosaccharides in vitro. Nutrients. 2020 Jun;12(6):1789.
28. He N, Yang Y, Wang H, Liu N, Yang Z, Li S. Unsaturated alginate oligosaccharides (UAOS) protects against dextran sulfate sodium-induced colitis associated with regulation of gut microbiota. J Funct Foods. 2021;83.
29. Wang Y, Wen R, Liu D, Zhang C, Wang ZA, Du Y. Exploring effects of chitosan oligosaccharides on the dss-induced intestinal barrier impairment in vitro and in vivo. Molecules. 2021;26(8).
30. Wan J, Zhang J, Chen D, Yu B, Huang Z, Mao X, et al. Alterations in intestinal microbiota by alginate oligosaccharide improve intestinal barrier integrity in weaned pigs. J Funct Foods. 2020;71(June):104040.
31. Liu P, Piao XS, Kim SW, Wang L, Shen YB, Lee HS, et al. Effects of chito-oligosaccharide supplementation on the growth performance, nutrient digestibility, intestinal morphology, and fecal shedding of Escherichia coli and Lactobacillus in weaning pigs. J Anim Sci. 2008;86(10):2609–18.
32. Zheng J, Cheng G, Li Q, Jiao S, Feng C, Zhao X, et al. Chitin oligosaccharide modulates gut microbiota and attenuates high-fat-diet-induced metabolic syndrome in mice. Mar Drugs. 2018;16(2).
33. Yang CM, Ferket PR, Hong QH, Zhou J, Cao GT, Zhou L, et al. Effect of chito-oligosaccharide on growth performance, intestinal barrier function, intestinal morphology and cecal microflora in weaned pigs. J Anim Sci. 2012;90(8):2671–6.
34. Wan J, Zhang J, Chen D, Yu B, Huang Z, Mao X, et al. Alginate oligosaccharide enhances intestinal integrity of weaned pigs through altering intestinal inflammatory responses and antioxidant status. RSC Adv. 2018;8(24):13482–92.
35. Zhao Y, Feng Y, Liu M, Chen L, Meng Q, Tang X, et al. Single-cell RNA sequencing analysis reveals alginate oligosaccharides preventing chemotherapy-induced mucositis. Mucosal Immunol. 2020;13(3):437–48.
36. Muanprasat C, Wongkrasant P, Satitsri S, Moonwiriyakit A, Pongkorpsakol P, Mattaveewong T, et al. Activation of AMPK by chitosan oligosaccharide in intestinal epithelial cells: Mechanism of action and potential applications in intestinal disorders. Biochem Pharmacol. 2015;96(3):225–36.
37. Shi L, Fang B, Yong Y, Li X, Gong D, Li J, et al. Chitosan oligosaccharide-mediated attenuation of LPS-induced inflammation in IPEC-J2 cells is related to the TLR4/NF-κB signaling pathway. Carbohydr Polym [Internet]. 2019;219(April):269–79. Available from: https://doi.org/10.1016/j.carbpol.2019.05.036
38. Asadpoor M, Ithakisiou GN, van Putten JPM, Pieters RJ, Folkerts G, Braber S. Antimicrobial Activities of Alginate and Chitosan Oligosaccharides Against Staphylococcus aureus and Group B *Streptococcus*. Front Microbiol. 2021;12(September).
39. Asadpoor M, Varasteh S, Pieters R, Folkerts G, Braber S. Differential effects of oligosaccharides on the effectiveness of ampicillin against Escherichia coli in vitro. PharmaNutrition. 2021;(February).
40. Khan S, Tøndervik A, Sletta H, Klinkenberg G, Emanuel C, Onsøyen E, et al. Overcoming drug resistance with alginate oligosaccharides able to potentiate the action of selected antibiotics. Velindre NHS Trust. 2012;
41. Fernandes JC, Tavaria FK, Soares JC, Ramos OS, João Monteiro M, Pintado ME, et al. Antimicrobial effects of chitosans and chitooligosaccharides, upon Staphylococcus aureus and Escherichia coli, in food model systems. Food Microbiol. 2008;25(7):922–8.

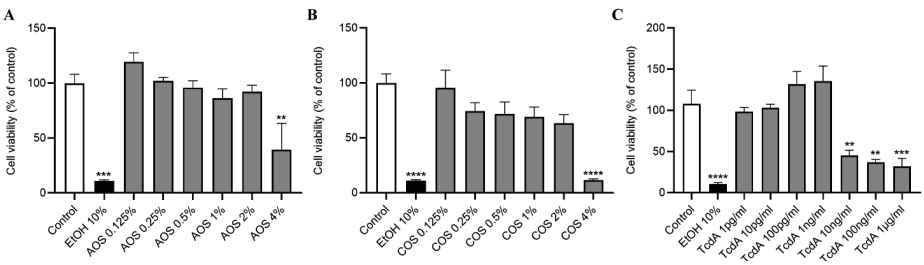
42. Rhoades J, Gibson G, Formentin K, Beer M, Rastall R. Inhibition of the adhesion of enteropathogenic Escherichia coli strains to HT-29 cells in culture by chito-oligosaccharides. Carbohydr Polym. 2006;64(1):57–9.
43. Jeon YJ, Park PJ, Kim SK. Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr Polym. 2001 Jan;44(1):71–6.
44. Sánchez, Mengíbar M, Rivera-Rodríguez G, Moerchbacher B, Acosta N, Heras A. The effect of preparation processes on the physicochemical characteristics and antibacterial activity of chitooligosaccharides. Carbohydr Polym. 2017;157:251–7.
45. Laokuldilok T, Potivas T, Kanha N, Surawang S, Seesuriyachan P, Wangtueai S, et al. Physicochemical, antioxidant, and antimicrobial properties of chitooligosaccharides produced using three different enzyme treatments. Food Biosci. 2017;18:28–33.
46. Kumar ABV, Varadaraj MC, Gowda LR, Tharanathan RN. Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of papain and Pronase , and their bactericidal action against Bacillus cereus and Escherichia coli. Biochem J. 2005;175:167–75.
47. Asadpoor M et al. Non-Digestible Oligosaccharides and Short Chain Fatty Acids and Their Toxins. Toxins (Basel). 2021;
48. Qu Y, Xu J, Zhou H, Dong R, Kang M, Zhao J. Chitin oligosaccharide (COS) reduces antibiotics dose and prevents antibiotics-caused side effects in Adolescent Idiopathic Scoliosis (AIS) patients with spinal fusion surgery. Mar Drugs. 2017;15(3):147–58.
49. Pinna A, Donadu MG, Usai D, Dore S, D'Amico-Ricci G, Boscia F, et al. In vitro antimicrobial activity of a new ophthalmic solution containing povidone-iodine 0.6% (IODIM®). Acta Ophthalmol. 2020;98(2):e178–80.
50. Kothary V, Doster RS, Rogers LM, Kirk LA, Boyd KL, Romano-Keeler J, et al. Group B *streptococcus* induces neutrophil recruitment to gestational tissues and elaboration of extracellular traps and nutritional immunity. Front Cell Infect Microbiol. 2017;7(FEB):1–13.
51. Fischer S, Ückert AK, Landenberger M, Papatheodorou P, Hoffmann-Richter C, Mittler AK, et al. Human peptide α-defensin-1 interferes with Clostridioides difficile toxins TcdA, TcdB, and CDT. FASEB J. 2020;34(5):6244–61.
52. Gigli S, Seguella L, Pesce M, Bruzzese E, D'Alessandro A, Cuomo R, et al. Cannabidiol restores intestinal barrier dysfunction and inhibits the apoptotic process induced by Clostridium difficile toxin A in Caco-2 cells. United Eur Gastroenterol J. 2017;5(8):1108–15.
53. Kyoon H, Young N, Ho S, Meyers SP. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. Int J Food Microbiol. 2002;74:65–72.
54. Benhabiles MS, Salah R, Lounici H, Drouiche N, Goosen MFA, Mameri N. Food Hydrocolloids Antibacterial activity of chitin , chitosan and its oligomers prepared from shrimp shell waste. Food Hydrocoll [Internet]. 2012;29(1):48–56. Available from: http://dx.doi.org/10.1016/j.foodhyd.2012.02.013
55. Zheng LY, Zhu JF. Study on antimicrobial activity of chitosan with different molecular weights. Carbohydr Polym. 2003 Dec;54(4):527–30.
56. Piotrowski M, Wultańska D, Obuch-Woszczatyński P, Pituch H. Fructooligosaccharides and mannose affect Clostridium difficile adhesion and biofilm formation in a concentration-dependent manner. Eur J Clin Microbiol Infect Dis. 2019 Oct;38(10):1975–84.
57. Hansen A, Alston L, Tulk SE, Schenck LP, Grassie ME, Alhassan BF, et al. The P2Y6 receptor mediates Clostridium difficile toxin-induced CXCL8/IL-8 production and intestinal epithelial barrier dysfunction. PLoS One. 2013;8(11):1–15.
58. Mahida YR, Makh S, Hyde S, Gray T, Borriello SP. Effect of Clostridium difficile toxin A on human intestinal epithelial cells: Induction of interleukin 8 production and apoptosis after cell detachment. Gut. 1996;38(3):337–47.
59. He D, Sougioultzis S, Hagen S, Liu J, Keates S, Keates AC, et al. Clostridium difficile toxin A triggers human colonocyte IL-8 release via mitochondrial oxygen radical generation. Gastroenterology. 2002;122(4):1048–57.
60. Shen A. Clostridium difficile toxins: Mediators of inflammation. J Innate Immun. 2012;4(2):149–58.

61. Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ. TEER Measurement Techniques for In Vitro Barrier Model Systems. J Lab Autom. 2015;20(2):107–26.
62. Firoozabadi TP, Shankayi Z, Izadi A, Firoozabadi SMP. Can Lucifer Yellow indicate correct permeability of biological cell membrane under an electric and magnetic field? Cell J. 2015;16(4):560–3.
63. Du T, Alfa MJ. Translocation of Clostridium difficile toxin B across polarized Caco-2 cell monolayers is enhanced by toxin A. Can J Infect Dis. 2004;15(2):83–8.
64. Xiong B, Liu M, Zhang C, Hao Y, Zhang P, Chen L, et al. Alginate oligosaccharides enhance small intestine cell integrity and migration ability. Life Sci. 2020;258(July):118085.
65. Yang JW, Tian G, Chen DW, Yao Y, He J, Zheng P, et al. Involvement of PKA signalling in anti-inflammatory effects of chitosan oligosaccharides in IPEC-J2 porcine epithelial cells. J Anim Physiol Anim Nutr (Berl). 2018;102(1):252–9.
66. Esposito G, Nobile N, Gigli S, Seguella L, Pesce M, d'Alessandro A, et al. Rifaximin improves Clostridium difficile toxin A-induced toxicity in Caco-2 cells by the PXR-dependent TLR4/MyD88/NF-κB pathway. Front Pharmacol. 2016;7(MAY):1–8.
67. Berkes J, Viswanathan VK, Savkovic SD, Hecht G. Intestinal epithelial responses to enteric pathogens: Effects on the tight junction barrier, ion transport, and inflammation. Gut. 2003;52(3):439–51.
68. Hecht G, Pothoulakis C, LaMont JT, Madara JL. Clostridium difficile toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. J Clin Invest. 1988;82(5):1516–24.
69. Chen ML, Pothoulakis C, Thomas Lamont J. Protein kinase C signaling regulates ZO-1 translocation and increased paracellular flux of T84 colonocytes exposed to Clostridium difficile toxin A. J Biol Chem. 2002;277(6):4247–54.
70. González-Mariscal L, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. Biochim Biophys Acta - Biomembr. 2008;1778(3):729–56.
71. Nusrat A, Von Eichel-Streiber C, Turner JR, Verkade P, Madara JL, Parkos CA. Clostridium difficile toxins disrupt epithelial barrier function by altering membrane microdomain localization of tight junction proteins. Infect Immun. 2001;69(3):1329–36.
72. Allam-Ndoul B, Castonguay-Paradis S, Veilleux A. Gut microbiota and intestinal trans-epithelial permeability. Int J Mol Sci. 2020;21(17):1–14.
73. Pongkorpsakol P, Satianrapapong W, Wongkrasant P, Steinhagen PR, Tuangkijkul N, Pathomthongtaweechai N, et al. Establishment of Intestinal Epithelial Cell Monolayers and Their Use in Calcium Switch Assay for Assessment of Intestinal Tight Junction Assembly. Methods Mol Biol. 2021;2367(April):273–90.
74. Wongkrasant P, Pongkorpsakol P, Ariyadamrongkwan J, Meesomboon R, Satitsri S, Pichyangkura R, et al. A prebiotic fructo-oligosaccharide promotes tight junction assembly in intestinal epithelial cells via an AMPK-dependent pathway. Biomed Pharmacother. 2020;129(April 2020):110415.
75. Olivier S, Leclerc J, Grenier A, Viollet MFB, Tamburini J. Ampk activation promotes tight junction assembly in intestinal epithelial caco-2 cells. Int J Mol Sci. 2019;20(20).
76. Krivan HC, Clark GF, Smith DF, Wilkins TD. Cell surface binding site for Clostridium difficile enterotoxin: Evidence for a glycoconjugate containing the sequence Ga1α1-3Galβ1-4GlcNAc. Infect Immun. 1986;53(3):573–81.
77. Rolfe RD, Song W. Purification of a functional receptor for clostridium difficile toxin a from intestinal brush border membranes of infant hamsters. Clin Infect Dis. 1993;16:S219–27.
78. Tucker KD, Wilkins TD. Toxin A of Clostridium difficile binds to the human carbohydrate antigens I, X, and Y. Infect Immun. 1991;59(1):73–8.

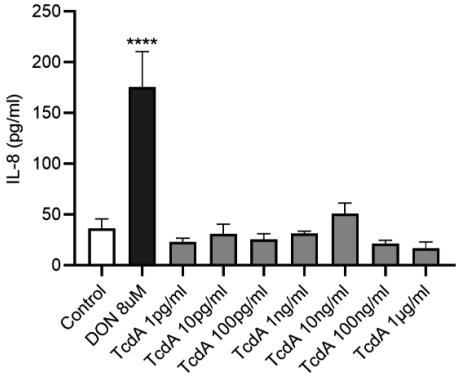
Supplementary figures



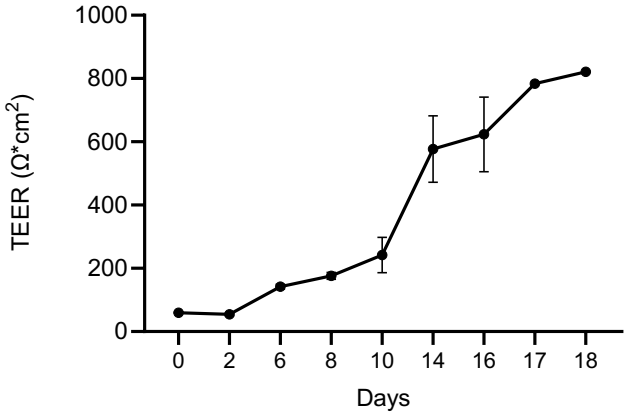
Supplementary figure 1. NDOs structures. The main components of AOS are 1,4-linked β-D-mannuronic acid and 1,4-linked α-L-guluronic acid (A) the main components of COS are 1,4-linked GlcNAc and 1,4-linked GlcN (B)



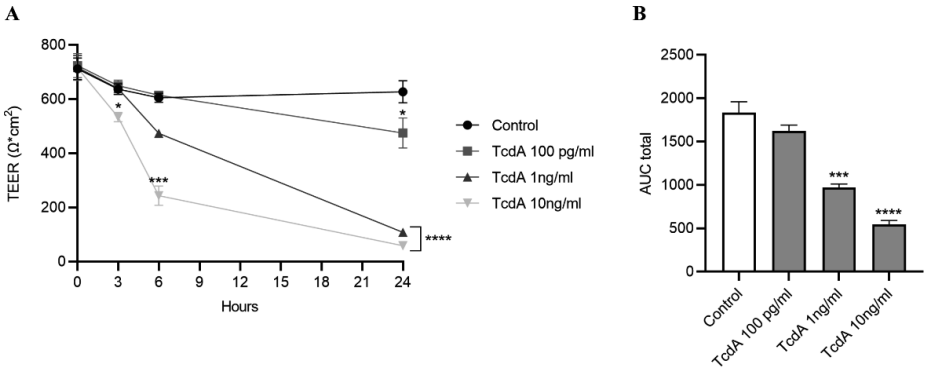
Supplementary figure 2. MTT cell viability assay. Caco-2 monolayers were grown in 96-well plates and exposed to (A) AOS and (B) COS 0.125%-4% for 48 h, or to (C) TcdA 1 pg/mL to 1 µg/mL for 48 hours. DMEM and ethanol 10% served as positive and negative control, respectively. Results are expressed as percentage of positive control as mean ± SEM of three independent experiments, each performed in triplicate (**P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001: significantly different from the unstimulated cells, as obtained using one-way ANOVA test).



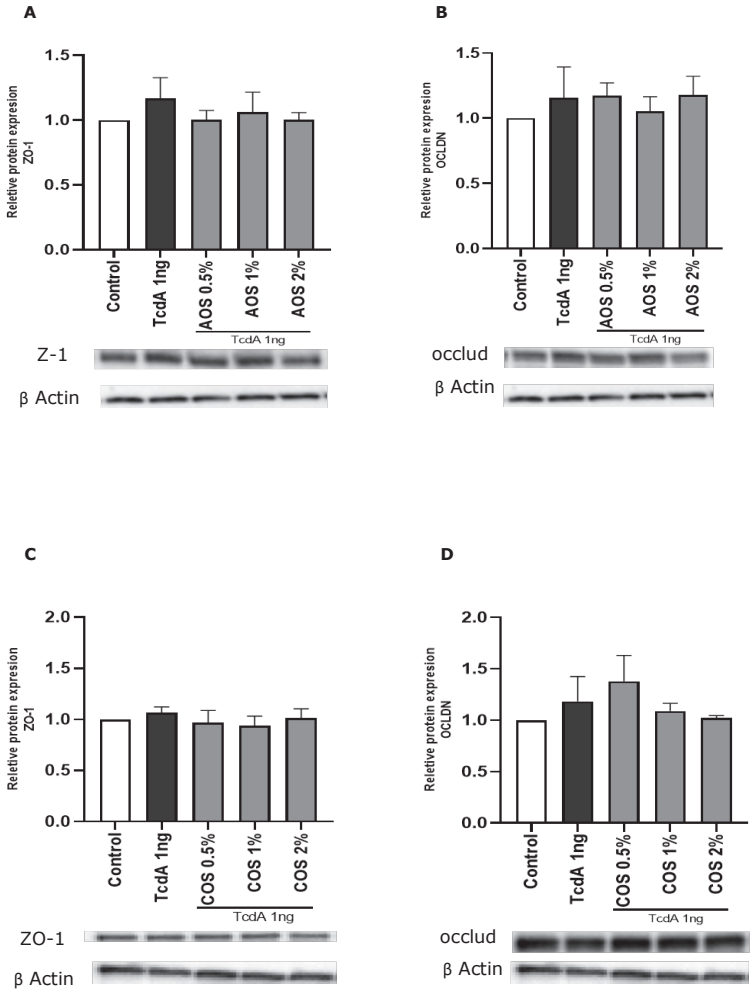
Supplementary figure 3. ELISA assay for CXCL8. Incubation of Caco-2 monolayers with ascending TcdA concentrations did not induce CXCL8 release. The toxin deoxynivalenol (DON) at a concentration of 8 µM and DMEM served as positive and negative control, respectively. Results are expressed as pg/mL of IL-8 released, as mean ± SEM of three independent experiments, each performed in triplicate (****P ≤ 0 .0001: significantly different from the unstimulated cells, as obtained using one-way ANOVA test).



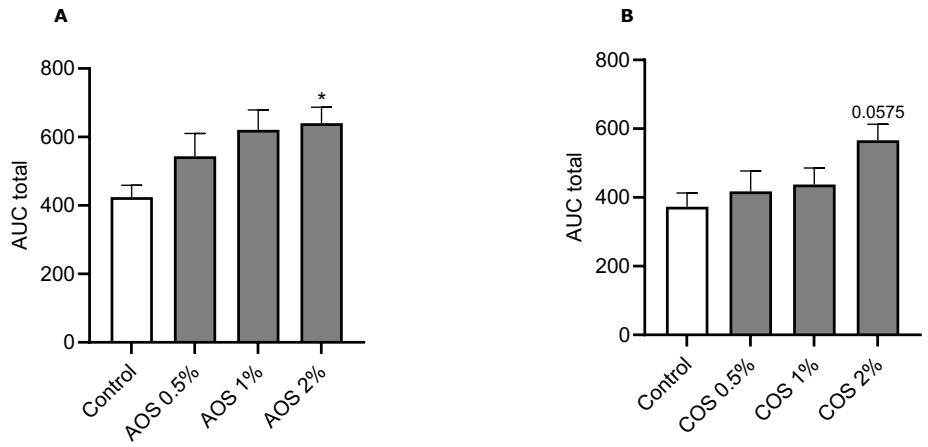
Supplementary figure 4. Caco-2 TEER trend. Caco-2 cells were grown in Transwell® permeable supports (0.4 µm pore size, 0.33 cm^2 cell growth area) for 17-21 days until formation of mature intercellular junctional contacts i.e., when TEER values reach a plateau with values in the range of ±700 Ω.cm^2. TEER values (Ω*cm^2) are expressed as mean ± SEM of three independent experiments, each performed in triplicate.



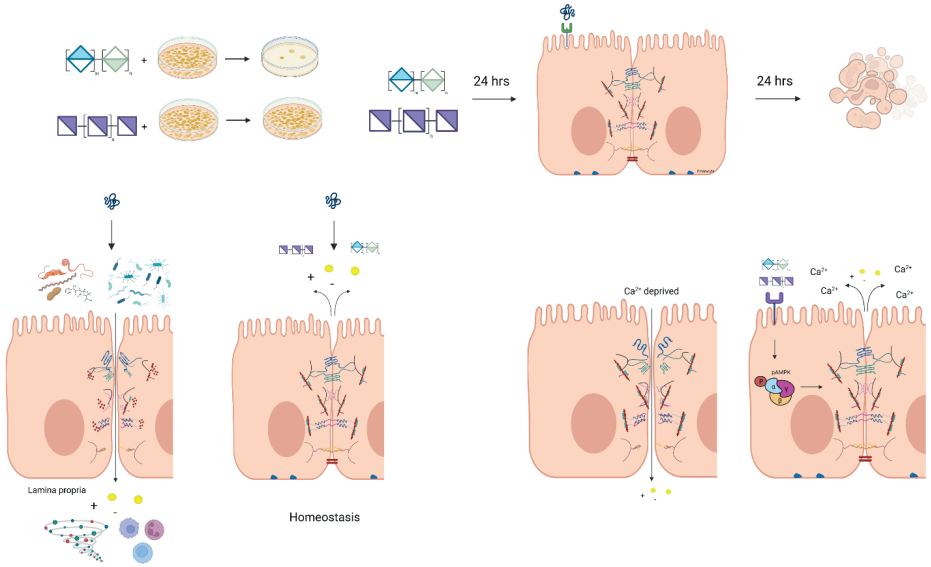
Supplementary figure 5. TEER trend of Caco-2 cells exposed to TcdA. Caco-2 cells grown in Transwell® inserts were exposed to TcdA 100 pg/mL, 1 ng/mL, and 10 ng/mL for 24 h. TEER values were measured prior to and after 6, 9, and 24 hours of TcdA exposure. TEER trend (Ω*cm^2) (A) and the total AUC for the time period of 24 h (B) are presented as mean ± SEM of three independent experiments, each performed in triplicate (*P ≤ 0.05, ***P ≤ 0.001, ****P ≤ 0 .0001: significantly different from the unstimulated cells, as obtained using one-way ANOVA test, with TEER values (Ω*cm^2) expressed as a percentage of the initial value).



Supplementary figure 6. Relative TJ protein expression in response to TcdA and TcdA/NDOs. The effect of TcdA 1 ng/mL on the protein levels of ZO-1 and occludin were quantified by Western blot analysis in Caco-2 cell lysates of cells that had been exposed to the toxin for 24 hours, with or without total supplementation (pre- and post-TcdA challenge treatment) with increasing AOS (A, B) or COS (C, D) concentrations. Results are expressed as mean ± SEM relative mRNA expression of three independent experiments, each performed in triplicate. No significant (*P ≤ 0.05) difference was observed compared to unstimulated cells, as obtained using one-way ANOVA test.



Supplementary figure 7. Calcium switch assay for AOS and COS. In order to examine whether the NDOs accelerate TJ re-assembly, Caco-2 cells grown on Transwell® inserts were pre-treated with increasing AOS/COS concentrations (24 h) prior to transient calcium deprivation with HBSS-EGTA to disrupt intercellular contacts. TEER values were measured during recovery (0, 2, 4, 6, 8, 10 h) in complete, calcium containing DMEM supplemented with either AOS (A) or COS (B). Results are expressed as the total area under the curve (AUC) calculated from TEER values for the time frame of 10h and expressed as the percentage of initial value as mean ± SEM of three independent experiments, each performed in triplicate (*P ≤ 0.05: significantly different from the unstimulated cells, as obtained using one-way ANOVA test).



Supplementary figure 8. Synopsis of the most important findings of this study. From top left to bottom right, based on the MIC assay results, AOS but not COS exert antigrowth activity against *C. difficile* bacteria. Next, the LDH cell cytotoxicity assay indicated that both NDOs significantly reduced TcdA-induced cytotoxicity when added to the Caco-2 monolayers 24 h prior to TcdA challenge. Furthermore, TcdA disrupts TJ functionality of intestinal epithelial cells, leading to impairment of the barrier's integrity which is evident *in vitro* by the increased ionic conductance (TEER measurements) and flux of paracellular tracers (LY transportation). Increased epithelial permeability permits gut microbes, allergens, and other pathogenic factors to permeate the monolayer, resulting in strong inflammatory and ismmune responses by IECs and immune cells present in the lamina propria. Pre-treatment with COS and AOS abrogates and alleviates, respectively, the TcdA-induced disruption of the Caco-2 monolayer, facilitating the maintenance of homeostasis. Finally, the Ca^{2+} switch assay demonstrated that both NDOs reinforce the intestinal barrier via accelerating the re-assembly of TJs, a process known to be mediated by stimulation of AMPK.

Ionic conductance: + -, LY: ●, TcdA: ❀, AOS: ◇◇, COS: ▭▭▭

Chapter 7

Fighting *Shigella* by Blocking its Disease-Causing Toxin

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Abstract

Shiga toxin is an AB5 toxin produced by the Shigella species while related toxins are produced by shiga toxin producing E. coli (STEC). Infection can lead to bloody diarrhea followed by the often fatal hemolytic uremic syndrome (HUS). In the present paper, we aimed for a simple and effective toxin inhibitor by comparing three classes of carbohydrate-based inhibitors: glycodendrimers, glycopolymers and oligosaccharides. We observed a clear enhancement in potency for the multivalent inhibitors, with the divalent and tetravalent compounds inhibiting in the millimolar and micromolar range respectively. However, the polymeric inhibitor based on galabiose was the most potent in the series exhibiting nanomolar inhibition. Alginate and chitosan oligosaccharides also inhibit shiga toxin and may be usable as a prophylactic during shigella outbreaks.

Keywords: Shiga toxin, HUS, Gb3 receptors, Glycopolymer, Oligosaccharides

1 Introduction

Bacterial dysentery or shigellosis has been identified as one of the major causes of mortality in children under 5 years of age (1). Shigellosis is caused by gram negative bacterium of four species of Shigella: S. dysenteriae, S. flexneri, S. boydii, and S. sonnei through the fecal-oral route. The pathology can include bloody diarrhea (hemorraghic colitis) followed by the often fatal hemolytic uremic syndrome (HUS). HUS can occur if the pathogen is also producing the Shiga toxin (Stx). The toxin is produced by S. dysenteriae serotype 1 but closely related toxins Stx1 and Stx2 are also produced by Shiga toxin producing E. coli (STEC) or enterohemorrhagic E. coli (EHEC), where Stx2 has been reported to cause the more severe infections (2). STEC outbreaks are mostly food-borne with the largest ever reported in Germany (2011), linked to sprout consumption (3).

The Shiga toxin is an AB5 toxin composed of the toxic A subunit and a pentameric B subunit that is responsible for the binding of the toxin to its cell surface receptor globotriaosylceramide (Gb3; Galα1-4Galβ1-4Glcβ1-ceramide, also known as CD77 or the Pk blood group antigen) (4). Each of the five B subunits can bind three Gb3 molecules simultaneously (5,6). After the initial bloody diarrhea the toxin enters the bloodstream by poorly understood mechanisms (7). The ample presence of Gb3 molecules in the kidney targets the toxin to this location. Once endocytosed, the toxin induces multiple signaling pathways leading to blockage of protein synthesis and induction of apoptosis (8) and HUS. STEC infections are treated with antibiotics although their use is controversial with respect to their ability to increase the risk of HUS (9). The recent emergence of toxin producing strains of S. flexneri and S. sonnei points towards increased future morbidity and mortality (10–19). As an alternative to antibiotics, synthetic molecules based on Gb3 have been explored as potential prophylactic treatment for STEC (20). Synsorb Pk, silicon dioxide coupled to synthetic Pk showed promising results in the trapping of toxins and preventing toxic effects on renal cells.(21) However, a subsequent clinical trial was unsuccessful at diminishing diarrhea-associated HUS possibly due to late administration of the drug to the GI tract while the toxin was already active systemically (22). Recommendations were made for intervention in the circulation. This approach was explored in several cases with antibodies, and nanobodies as recently summarized (23). Smaller dendritic molecules were also explored in this respect. The soluble STARFISH inhibitor with decavalent display of Gb3 trisaccharide, reported by Bundle et al. exhibited subnanomolar inhibition of Stx1, with large potency gains over the divalent analogue and the Pk trisaccharide itself (24). A modification of the STARFISH named DAISY was observed to be effective against both Stx1 and Stx2 with nanomolar inhibition and in vivo activity in EHEC orally infected mice by subcutaneous injection 24 h after infection (25). Several related SUPERTWIG structures (based on Gb3 conjugated to carbosilane dendrimers), developed by Nishikawa et al. were also identified as effective neutralizers of Stx with a dependency on their valency and structure (26). A hexavalent structure provided protection after intravenous injections starting 3 days after oral infection.

In the present paper, three classes of carbohydrate-containing structures were investigated: dendritic synthesized multivalent inhibitors, glycopolymers and natural oligosaccharides. As the ligand we chose to explore the potential of the disaccharide (Galα1-4Galβ; galabiose) as a possible monovalent alternative to Gb3 based inhibitors. The intention here was to explore what the minimal structural requirements for potent toxin inhibition would be, by minimizing the ligand and the multivalent scaffold. For the dendrimers, ease of preparation was central to the selection of di-and tetravalent dendrimers utilized. Polymeric scaffolds were selected for potency comparison. For the polymer scaffold, hyperbranched polyglycerol (hPG) were used for their easy synthesis, high functionalization, biocompatibility and low in vivo toxicity.(27) A polyalkyne and a polyazide variant of hPG was prepared for their conjugation by employing the copper-catalyzed alkyne azide cycloaddition (CuAAC) conjugations. In addition to the synthesized compounds, commercially available oligosaccharides: non-toxic food grade alginate, chitosan, fructo- and galacto-oligosaccharides (AOS, COS, GOS, FOS)(28) were tested for Stx inhibition. These could serve as an even more viable practical alternative that could be part of a preventative food-based approach during outbreaks with a focus on the gastrointestinal phase of the toxin producing Shigella pathogenicity.

2 Material and methods

Plasmid construction, protein expression and protein purification

The Stx1B expression plasmid was constructed by using the Gateway® recombinant cloning kit (ThermoFisher, Spain). Briefly, a synthetic DNA cassette (Invitrogen, Spain) that encodes residues 21-89 of Stx1B (GenBank: AAA98348.1) with C-terminal 6×His tag was first inserted into an entry plasmid pENTR1A through restriction sites Dra I and Xho I. The resultant plasmid Stx1B-His-pENTR1A together with a destination vector pDEST 14 was further subjected to the Gateway® LR Clonase cloning reaction to achieve the final protein expression construct Stx1B-His-pDEST14 following the manufacturer's instructions.

Protein expression was carried out using E. coli BL21 cells transformed with the plasmid Stx1B-His-pDEST14. The above E. coli cells were grown in LB broth media containing 100 mg/ml ampicillin at 37 °C until OD450 reached 0.6 and then IPTG was added to the culture at final concentration of 1 mM to induce the expression of recombinant protein at room temperature for 16 h. At the end of IPTG induction, E. coli cells were immediately lysed in the culture using B-PER direct bacterial protein extraction Kit (ThermoFisher, Spain) with the supplement of protease inhibitor (EDTA free), DNase I and lysozyme following the manufacturer's instructions. After centrifugation 10 min at 12000 rpm at 4 °C, the supernatant was collected for further protein purification.

Protein purification was performed using a home-made column packed with HisPur™ Ni-NTA Resin (ThermoFisher, Spain) according to the manufacturer's instructions. After the column was washed with an equilibration buffer (20 mM sodium phosphate, 300 mM

sodium chloride, 10 mM imidazole, pH 7.4), it was then loaded with the above supernatant containing 6×His tagged Stx1B at 4 °C for 1h. Unbound proteins were removed from the column by using a washing buffer (20 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole, pH 7.4). Finally, 6×His tagged Stx1B was eluted from the column using an elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, 300 mM imidazole, pH 7.4) and later confirmed by SDS-PAGE (Supplementary figure 1). Before applied for the later binding assay, imidazole residues in the eluted proteins were removed using a 10 kDa molecular weight cut off protein concentrators (ThermoFisher, Spain) and a buffer containing 20 mM sodium phosphate, 500 mM sodium chloride.

Shiga toxin inhibition assay *(Stx1B ELISA)*

A 96-well plate (Nunc PolySorp™) was coated with a solution of FSL-GB3 (50 µL, 2 µg/mL) in phosphate buffered saline (PBS) for 3 hours at room temperature. Unattached GB3 was removed by washing with PBS (0.2% BSA) and the remaining binding sites of the surface were blocked with BSA (1%) for 1 h, followed by washing with PBS (0.2% BSA). Samples of Stx1B (50 µL, 0.1 µg/mL) and inhibitor were transferred to the GB3-coated plate and incubated at r.t. for 1 h followed by washing with PBS (0.2% BSA). HisProbe-HRP (4 mg/mL, 1:2000 dilution, 100 µL/well) was incubated for 0.5 h followed by washing with PBS (0.2% BSA) (49). HRP activity was measured by using 1-Step™ Ultra TMB-ELISA substrate solution (100 µL/well) for a maximum of 10 min. After quenching with H2SO4, the absorbance in each well was measured at 450 nm. Compounds 3, 4, 5, 6, 8 and 9 were tested at least twice in duplicate or triplicate whereas compound 1e was tested once. Inhibition data from the experiments were averaged and fitted in GraphPad Prism 8.3.0 with a non-fixed Hill-slope.

Cell culture

Human colonic epithelial T84 cells (ATCC® CCL-248™) were cultured in Dulbecco's modified eagle's medium: Nutrient mixture F-12 (DMEM / F-12; Gibco, Invitrogen, Carlsbad, CA, USA) (1:1) supplemented with 10 % fetal calf serum (FCS; Gibco), penicillin (100 U / mL) and streptomycin (100 g / mL) (Biocambrex) and maintained at 37°C in a humidified incubator with 5 % CO2. T84 cells were grown on plastic culture flasks (75 cm2) at a density of 3*106 cells / ml. After 7 days, T84 cells were seeded on 0.3 cm2 high pore density polyethylene terephthalate membrane transwell inserts with 0.4 µm pores (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) placed in a 24-well plate (density of 3*105 cells/ insert) or in 96-well microtiter plates (Costar 3614, Corning, NY, USA) at a density of 3*104 cells/well. Cells were passaged by addition of trypsin ethylene diamine tetra-acetic acid (EDTA) at 100 % confluency every week. The experiments were performed at passage number 51- 55 on fully confluent monolayers with transepithelial electrical resistance (TEER) values > 1000 Ω · cm2.

Oligosaccharides

Fructo-oligosaccharides (FOS) isolated from chicory were obtained from Orafti (Wijchen, The Netherlands) (purity > 97 %). Galacto-oligosaccharides (GOS) (Vivinal® GOS Powder,

purity >70%) produced from lactose were provided by FrieslandCampina (Amersfoort, The Netherlands). Alginate Oligosaccharides (AOS) prepared by degradation of algin (purity > 85 %) and chitosan oligosaccharides (COS) derived from rich marine biological sources (shrimp & crab shells) (purity > 90%) were both purchased from BZ Oligo Biotech Co., Ltd. (Qingdao, Shandong, China). All oligosaccharide solutions were freshly prepared through dissolution in DMEM / F12 and their pH was adjusted to pH=7.2 – 7.4.

Transepithelial electrical resistance (TEER) measurement

For evaluating the epithelial integrity of the T84 monolayer, TEER values were measured using a Millicell-ERS Volt-Ohm-meter (Millipore, Temecular, CA, USA). As described above, T84 cells were seeded at a density of 3*105 cells/insert and cultured for 3 weeks. The inserts were placed in a 24-well plate with 300 µL medium at the apical compartment and 700 µL medium at the basolateral compartment. Different concentrations of the Glycopolymer **9** (1, 10 and 100 nM) were added to the apical compartment of the transwell inserts. Transwell inserts of T84 cells, treated with medium were considered as control group. The TEER values were measured before and 24 h after exposure to different concentrations of Glycopolymer 9 incubated at 37 °C in 5% CO2. The transepithelial electrical resistance was expressed as $\Omega \cdot cm2$.

Viability – MTT Assay

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay (Sigma-Aldrich, St. Louis, Mo, USA). T84 cells were seeded on a flat bottomed 96-well plate at a density of 3*104 cells/well and grown for 7 days until they reached 100 % confluency. Thereafter, cells were exposed to three concentrations of glycopolymer 9 (1, 10 and 100 nM) and 10% ethanol was used as positive control. The wells with medium (no treatment) were considered as control group. After 24 h incubation at 37 °C in 5 % CO2, the medium was removed and 120 µL of MTT working solution [20 µL MTT (5 mg/mL) and 100 µL medium] was added to each well and incubated for 2 h under the same conditions. Finally, DMSO was added to lyse the cells and dissolve the purple blue sediment. After 5 min of mild shaking, the absorbance value of each well was measured at 595 nm using a Glomax Discover microplate reader. The viability of the T84 cells was calculated based on the following equation: (mean absorbance of treatment cells / mean absorbance of control cells)*100.

Statistical analysis

Data were reported as mean values ± SEM of three independent experiments (n=3) routinely performed in triplicate (3 wells/condition). Results were analyzed using Prism 8.0 GraphPad Software (GraphPad, San Diego, CA, USA). Statistical significance was determined using one-way ANOVA followed by Bonferroni post-hoc test. Differences were considered statistical significant when $P < 0.05$.

Chemistry

Chemicals were obtained from commercial sources and were used without further purification unless noted otherwise. The solvents were obtained as synthesis grade and stored on molecular sieves (4 Å). TLC was performed on Merck precoated Silica plates. Spots were visualized by UV light and 10% H2SO4 in MeOH . Microwave reactions were carried out in a Biotage microwave Initiator (300W, Uppsala, Sweden). The microwave power was limited by temperature control once the desired temperature was reached. Sealed vessels of 2-5 mL and 10-20 mL were used. 1H NMR, HSQC, COSY (600 MHz) and 13C (151 MHz) were performed on a Bruker 600 spectrometer. Infrared (IR) spectroscopy was performed using Universal Attenuated Total Reflectance (UATR) accessory of Perkin Elmer Spectrum Two FT-IR. High resolution mass spectrometry (HRMS) analysis was recorded using an Agilent 6560 Ion Mobility Q-TOF LC/MS instrument. Analytical HPLC and Preparative HPLC runs were performed on a Shimadzu 20A HPLC system. Analytical HPLC was performed using a Dr Maisch GmBh C18-AQ column (5 µm) at a flow rate of 0.5 mL/min. The used buffers were H2O (Buffer A) and CH3CN (Buffer B). Runs were performed using a standard protocol: 2 – 100 % gradient buffer B in 35 min, UV-absorption was measured at 254 nm. For Preparative HPLC, a Waters XBridge BEH Prep Amide column (5 µm, 250×10 mm) at a flow rate of 2.4 mL/min was used. Runs were performed using a standard protocol: 95 – 50 % gradient buffer B in 60 min. UV-absorption was measured at 254 nm and 210 nm. Commercial Oligosaccharides: AOS (food grade) was purchased from Qingdao Bz Oligo Biotech Co. Ltd. COS (9012-76-4) with a degree of deacetylation ≥95%. GOS was purchased from Friesland Campina (Vivinal® GOS powder, 69%).Bis-alkyne 2a(29) and Tetra-alkyne 2c(30) were synthesized according to the reported procedures, with the spectral data in agreement with the reported values.

General CuAAC Procedure for the Synthesis of Multivalent Galabiose Compounds 3, 4, 5, 6

All tested compounds were >95% pure by HPLC. The alkyne (2a, 2b, 2c, 2d, 1 equiv) was dissolved in DMF followed by the addition of the ligand 1d (1.2 equiv). Copper sulphate pentahydrate (0.1 equiv) was dissolved in water separately and added to the reaction mixture. Sodium ascorbate (0.3 equiv) was also dissolved in water separately and added to the reaction mixture. The reaction was performed at 80°C in the microwave for 1 h. The reaction mixture was extracted using EtOAc and water, followed by column purification (6% MeOH in DCM) to obtain the purified compound which were further subjected to deacetylation as described below.

General procedure for the deacetylation reaction

The peracetylated compound was dissolved in anhydrous methanol, followed by addition of a catalytic amount of an aqueous NaOH solution (1 M) and stirred at room temperature. The reaction was monitored by TLC. Upon completion of the reaction, the mixture was neutralized by the addition of Dowex marathon resin. The solvent was evaporated and the crude mixture was purified by preparative HPLC to obtain the pure product (1e, 3, 4, 5, 6) in >80% yields

Azide 1c. Compound **1a** (2.00 g, 3.13 mmol, 1 equiv), diphenyl sulfoxide (1.2 g, 6.0 mmol, 2.6 equiv), and 2,4,6-tris-tert-butylpyrimidine (2.232 g, 9.0 mmol, 3.0 equiv) were dissolved in anhydrous DCM (45 mL) under an atmosphere of argon. Activated molecular sieves (3 Å) were added. The solution was then cooled to -40°C and trifluoromethanesulfonic anhydride (500 µL, 3 mmol, 1.3 equiv) was added. The mixture was stirred for 10 min and galactose acceptor **1b** (2.261 g, 4.373 mmol, 1.4 equiv) was added as a solution in anhydrous DCM (40 mL). The reaction was stirred for ca. 1.5 h at -40 °C and then quenched by addition of triethylamine (5 mL, excess). The mixture was diluted with DCM (100 mL) and washed with 1M HCl and saturated aqueous sodium bicarbonate. The organic layer was dried with NaSO4, filtered and concentrated in vacuo. Purification was done by column chromatography (0-20% EtOAc in petroleum ether) to yield the product (1.655 g, 1.58 mmol, 50%). 1H NMR (600 MHz, CDCl3) δ 8.09 (dd, J = 8.1, 1.4 Hz, 2H, 2xCH Bz ortho), 8.04 (dd, J = 8.3, 1.3 Hz, 2H, 2xCH Bz ortho), 7.93 (dd, J = 8.2, 1.4 Hz, 2H, 2xCH Bz ortho), 7.62 – 7.56 (m, 1H, CH Bz para), 7.50 (dddd, J = 8.8, 6.1, 3.0, 1.3 Hz, 2H, 2xCH Bz para), 7.46 (t, J = 7.8 Hz, 2H, 2xCH Bz meta), 7.36 (td, J = 7.8, 3.7 Hz, 4H, 4xCH Bz meta), 5.69 (dd, J = 10.5, 8.6 Hz, 1H, H-2), 5.46 (dd, J = 10.5, 2.4 Hz, 1H, H-3), 5.02 (d, J = 3.1 Hz, 1H, H'-1), 4.83 – 4.76 (m, 2H, H-1, H-6a), 4.68 (dd, J = 12.1, 7.3 Hz, 1H, H-6b), 4.41 (d, J = 2.5 Hz, 1H, H-4), 4.38 (d, J = 2.4 Hz, 1H, H'-4), 4.24 – 4.16 (m, 3H, H'-2, H-5, H'-6a), 4.12 – 4.07 (m, 2H, H'-3, H'-5,), 4.05 (dd, J = 12.7, 1.9 Hz, 1H, H'-6b), 1.03 (s, 9H, SiC(CH3)3), 1.00 (s, 9H, SiC(CH3)3), 0.97 (s, 9H, SiC(CH3)3), 0.86 (s, 9H, SiC(CH3)3), 0.19 (s, 3H, SiCH3), 0.17 (s, 3H, SiCH3), 0.08 (s, 3H, SiCH3), 0.04 (s, 3H, SiCH3). 13C NMR (151 MHz, CDCl3) δ 166.15 (C=O, Bz), 165.82 (C=O, Bz), 165.12 (C=O, Bz), 133.60 (CH, Bz para), 133.36 (CH, Bz para), 133.21 (CH, Bz para), 130.01 (2xCH, Bz ortho), 129.83 (2xCH, Bz ortho), 129.80 (2xCH, Bz ortho), 129.78 (C Bz), 128.94 (C Bz), 128.67 (C Bz), 128.57 (2xCH, Bz meta), 128.43 (2xCH, Bz meta), 128.38 (2xCH, Bz meta), 101.45 (C'-1), 88.38 (C-1), 75.69 (C'-5), 75.05 (C'-4), 75.04 (C-4), 73.17 (C-3), 70.94 (C'-3), 70.04 (C'-2), 69.17 (C'-5), 68.68 (C-2), 67.02 (C'-6), 64.12 (C-6), 27.46 (SiC(CH3)3), 27.36 (SiC(CH3)3), 26.23 (SiC(CH3)3), 26.21 (SiC(CH3)3), 23.41 (2xSiC(CH3)3), 18.36 (SiC(CH3)3), 18.22 (SiC(CH3)3), -3.91 (SiCH3), -4.23 (SiCH3), -4.35 (SiCH3), -4.66 (SiCH3).

Azide 1d. Compound **1c** (1655 mg, 1.58 mmol 1 equiv) was solved in MeOH (30 mL) and an excess of K2CO3 was added. The reaction was stirred at r.t. for 16 h, then filtered and concentrated in vacuo. The crude product was dissolved in EtOAc and extracted with aqueous NaHCO3 and the organic layer was separated, dried with NaSO4, filtered and concentrated again in vacuo. The product was purified using column chromatography (10-50% EtOAc in petroleum ether) to yield the product (1034 mg, 1.41 mmol, 89%).The debenzoylated product (332 mg, 0.452 mmol, 1 equiv) was dissolved in DCM (5 mL) and HF-Pyridine (70%, 0.4 mL) was added dropwise at r.t. under continuous argon flow. The reaction was stirred for 2 h and quenched by addition of solid CaCl2 (99 mg, 0.904 mmol, 2 equiv) Pyridine (10 mL, 124 mmol, 272 equiv), Ac2O (5 mL, 53 mmol, 117 equiv) and DMAP (3 mg, 0.02 mmol, 0.05 equiv) were added. The reaction was stirred for 16 h at r.t. then diluted with EtOAc, and washed with saturated, aqueous K2CO3. The organic phase was dried with NaSO4, filtered, and concentrated in vacuo. The product was purified using

silica gel flash chromatography using a gradient of 40-100% EtOAc in petroleum ether (154 mg, 0.232 mmol, 51% (over 3 steps)). The spectral data was in accordance with published data (50).1H NMR (600 MHz, CDCl3) δ 5.53 (dd, J = 3.4, 1.3 Hz, 1H, H'-4), 5.33 (dd, J = 11.0, 3.4 Hz, 1H, H'-3), 5.16 (dd, J = 11.0, 3.7 Hz, 1H, H'-2), 5.12 (dd, J = 10.7, 8.6 Hz, 1H, H-2), 4.99 (d, J = 3.7 Hz, 1H, H'-1), 4.83 (dd, J = 10.7, 2.7 Hz, 1H, H-3), 4.63 (d, J = 8.6 Hz, 1H, H-1), 4.45 (ddd, J = 7.6, 5.8, 1.3 Hz, 1H, H'-5), 4.40 (dd, J = 11.4, 6.9 Hz, 1H, H-6a), 4.12 (dd, J = 11.3, 6.1 Hz, 1H, H-6b), 4.10 – 4.04 (m, 3H, H'6ab; H-4), 3.87 (t, J = 6.5 Hz, 1H, H-5), 2.09 (s, 3H, CH3 Ac), 2.07 (s, 3H, CH3 Ac), 2.05 (s, 3H, CH3 Ac), 2.04 (s, 3H, CH3 Ac), 2.04 (s, 3H, CH3 Ac), 2.00 (s, 3H, CH3 Ac), 1.95 (s, 3H, CH3 Ac). 13C NMR (151 MHz, CDCl3) δ 170.45 (C=O, Ac), 170.37 (C=O, Ac), 170.32 (C=O, Ac), 170.27 (C=O, Ac), 169.98 (C=O, Ac), 169.66 (C=O, Ac), 168.90 (C=O, Ac), 98.97 (C'-1), 88.11 (C-1), 76.27 (C-4), 74.03 (C-5), 72.40 (C-3), 68.30 (C'-2), 67.88 (C-2), 67.67 (C'-4), 67.18 (C'-5), 67.07 (C'-3), 61.81 (C-6), 60.42 (C'-6), 20.76 (CH3, Ac), 20.62 (CH3, Ac), 20.58 (CH3, Ac), 20.54 (CH3, Ac), 20.51 (CH3, Ac), 20.47 (CH3, Ac).

Azide 1e. Compound **1d** was deprotected using the general procedure described above to obtain the final compound 1e in 90% yield. The spectral data was in accordance with published data (51). 1H NMR (600 MHz, MeOD) δ 4.94 (d, J = 3.7 Hz, 1H, H'-1), 4.52 (d, J = 8.4 Hz, 1H, H-1), 4.18 (t, J = 6.2 Hz, 1H, H-5), 3.99 (d, J = 3.0 Hz, 1H, H-4), 3.88 (d, J = 3.2 Hz, 1H, H'-4), 3.85 – 3.71 (m, 6H, H'-2, H'-3, H'-5, H'-6ab, H-6a), 3.65 (dd, J = 11.2, 5.1 Hz, 1H, H-6b), 3.52 (dd, J = 10.0, 2.9 Hz, 1H, H-3), 3.41 (dd, J = 10.3, 1.9 Hz, 1H, H-2). 13C NMR (151 MHz, MeOD) δ 101.43 (C'-1), 91.39 (C-1), 78.37 (C-5), 76.70 (C-4), 73.43 (C-3), 71.62 (C'-5), 71.02 (C-2), 69.83 (C'-4), 69.66 (C'-2), 69.20 (C'-3), 61.30 (C-6), 59.70 (C'-6).

Compound 2b. Methyl 3,5-bis(2-(boc-amino)ethoxy)benzoate (110 mg, 0.25 mmol, 1 equiv) was prepared as reported(30) and dissolved in 1:1 TFA:DCM and stirred at r.t. for 2 h before concentrating in vacuo. The residue was dissolved in DCM (10 ml) and TEA (139 µL, 1 mmol, 4 equiv) was added and the mixture was left stirring for 5 minutes at r.t. before cooling to 0 °C. Propargyl chloroformate (55 µL, 0.55 mmol, 2.2 equiv.) was added dropwise and the reaction was allowed to slowly warm up to r.t. and was left stirring for 16 h. The reaction was diluted with an excess of DCM and 1M aq. HCl and the organic layer was collected, dried with NaSO4, filtrated and concentrated in vacuo. The compound was purified by column chromatography using a gradient of 0-50% EtOAc in petroleum ether and with 1% TFA yielding the free acid (89 mg, 0.22 mmol, 88%). 1H NMR (600 MHz, Methanol-d4) δ 7.20 (d, J = 2.4 Hz, 2H, 2xCHarom-2,6), 6.79 (d, J = 2.5 Hz, 1xCHarom-4), 4.68 (d, J = 2.4 Hz, 4H, 2xCH2, propargyl), 4.07 (t, J = 5.5 Hz, 4H, 2xOCH2), 3.41 (t, J = 5.5 Hz, 4H, 2xNCH2), 2.89 (t, J = 2.5 Hz, 2H, 2xC≡CH). 13C NMR (151 MHz, Methanol-d4) δ 169.61 (C=O, acid), 161.30 (CO, aromatic), 158.13 (2xC=O, carbamate), 134.17 (Carom-COOH), 109.32 (2xCHarom-2,6), 107.26 (CHarom-4), 79.44 (2xC≡CH), 75.80 (2xC≡CH), 68.11 (2xOCH2), 53.20 (2xCH2, propargyl), 41.43 (2xNCH2). HR-ESI-TOF/MS (m/z): [M+Na]+ calcd. for C19H20N2O8, 427.1117; found, 427.1116.

Compound 2d. To a solution of **2a** (16.4 mg, 82 µmol) in DCM (1 mL) was added (COCl)2 (31 µL, 246 µL, 3 eq.) and DMF (10 µL). After stirring at r.t. for 1.5 h, the mixture was concentrated. The resulting residue was coevaporated with 10 mL anhydrous toluene and then redissolved in DCM (1 mL), and cooled to 0 ºC. A solution of pyridine (1 mL), DCM (1 mg) and dodecane-1,12-diamine (10 mg) was added slowly to the reaction flask. The resulting mixture was stirred at r.t. overnight. Solvents were removed and the residue was partitioned between EtOAc and water. The organic layer was separated, washed with brine (1 x), dried with anhydrous MgSO4, filtered, and concentrated. The residue was purified by silica gel flash chromatography and yielded product (20 mg, 80%). 1H NMR (600 MHz, Acetone-d6) δ 7.57 (t, J = 5.9 Hz, 2H, NH), 7.02 (s, 4H, 4xCH arom), 6.64 (s, 2H, 2xCH arom), 4.71 – 4.67 (m, 8H, (4xOCH2), 3.24 (q, J = 6.7 Hz, 4H, 4xCH propargyl), 2.97 (s, 4H, 2xNHCH2), 1.92 (s, 4H, 2x CH2), 1.46 (t, J = 7.1 Hz, 4H, 2x CH2), 1.28 – 1.02 (m, 12H, 6x CH2). 13C NMR (151 MHz, Acetone-d6) δ 165.64 (2xC=O), 158.77 (4xCO, arom), 137.57 (2xC-CONH), 106.68 (4xCH), 104.54 (2xCH), 78.54 (4xCCH, alkyne), 76.38 (4xCCH, alkyne), 55.70 (4xOCH2), 39.64 (2xN-CH2), 29.32 (CH2), 29.30 (CH2), 29.17 (CH2), 29.15 (CH2), 29.04 (CH2), 29.01 (CH2), 28.90 (CH2), 28.77(CH2), 28.64 (CH2). HR-ESI-TOF/MS (m/z): [M+H]+ calcd. for C38H44N2O6, 625.3277; found, 625.3302.

Compound 3. 1H NMR (600 MHz, Deuterium Oxide) δ 8.23 (s, 2H, triazole), 7.07 (dd, J = 2.3, 0.9 Hz, 2H, 2x CHarom-2,6),), 6.68 (td, J = 2.3, 0.8 Hz, 1H, 1x CHarom-4), 5.58 (dd, J = 9.0, 0.8 Hz, 2H, 2x H-1), 5.18 (s, 4H, 2x OCH2), 4.90 (d, J = 4.0 Hz, 2H, 2x H'-1), 4.29 (t, J = 6.4 Hz, 2H, 2x H-5), 4.20 (t, J = 9.5 Hz, 2H, 2x H-2), 4.07 (d, J = 3.2 Hz, 2H, 2x H'-5), 3.96 – 3.68 (m, 14H, 2x H'-4, 2x H'-2, 2x H'-6, 2x H-4, 2x H'-3, 2x H-3), 3.64 – 3.55 (m, 4H, 2x H-6).

13C NMR (151 MHz, Deuterium Oxide) δ 174.24 (-COOH), 158.33 (2xCarom-3), 143.27 (2x -OCH2-C), 139.24 (COOH-C), 125.28 (2x N-CH), 108.97 (2x CHarom-2,6),), 105.72 (1x CHarom-4), 100.48 (2xC'-1), 88.10 (2xC-1), 78.28 (2xC'-5), 77.24 (2xC-4), 72.69 (2xC'-3), 70.90 (2xC-5), 69.61 (2xC-3), 69.05 (2xC'-2), 68.98 (2xC'-4), 68.63 (2xC-2), 61.39 (2x OCH2), 60.54 (2xC'-6) , 60.06 (2xC-6). HR-ESI-TOF/MS (m/z): [M+Na]+ calcd. for C37H52N6O24, 987.2930; found, 987.2934.

Compound 4. 1H NMR (600 MHz, Deuterium Oxide) δ 8.23 (s, 2H, triazole), 7.07 (s, 2H, 2x CHarom-2,6),), 6.66 (s, 1H, 1x CHarom-4), 5.66 (d, J = 9.0 Hz, 2H, 2x H-1), 5.20 (d, J = 2.4 Hz, 4H, 2xNH-CO-CH2), 5.03 (d, J = 4.0 Hz, 2H, 2x H'-1), 4.41 (t, J = 6.5 Hz, 2H, 2x H-5), 4.31 (t, J = 9.6 Hz, 2H 2x H-2), 4.20 (d, J = 3.0 Hz, 2H, 2x H-3), 4.13 (t, J = 5.1 Hz, 4H, 2xCONH-CH2-CH2), 4.07 – 3.84 (m, 14H, 2x H'-4, 2x H'-3, 2x H'-2, 2x H'-5, 2x H-4, 2x H-6), 3.78 – 3.68 (m, 4H, 2x H'-6), 3.52 (t, J = 5.2 Hz, 4H, 2xCONH-CH2-CH2). 13C NMR (151 MHz, Deuterium Oxide) δ 157.89 (2xCarom3,5), 125.11 (2x N-CH), 108.29 (2x CHarom-2,6),), 100.47 (2xC'-1), 88.08 (2xC-1), 78.23 (2xC'-5), 77.20 (2xC-4), 72.68 (2xC'-3), 70.89 (2xC-5), 69.59 (2xC-3), 69.06 (2xC'-2), 68.97 (2xC'-4), 68.62 (2xC-2), 67.18 (2x OCH2), 60.53 (2xC'-6), 60.00 (2xC-6), 57.63 (2xNHCO-O-CH2), 40.09 (2x OCH2-CH2).

HR-ESI-TOF/MS (m/z): [M+Na]+ calcd. for C43H62N8O28, 1161.3571; found, 1161.3574.

Compound 5. 1H NMR (600 MHz, Deuterium Oxide) δ 8.26 (s, 4H, triazole), 7.06 (s, J = 1.9 Hz, 2H, 2x CHarom-2,6), 6.70 (s, 1H, 1x CHarom-4), 6.62 (d, J = 2.2 Hz, 4H, 4x CHarom-2',6'), 6.44 (s, 2H, 2x CHarom-4'), 5.64 (d, J = 9.0 Hz, 4H, 4x H-1), 5.00 (d, J = 4.0 Hz, 4H, 4x H'-1), 4.95 (s, 8H, 4x triazole-CH2-), 4.35 (t, J = 6.4 Hz, 4H, 4x H-5), 4.29 (t, J = 9.6 Hz, 4H, 4x H-2), 4.17 (d, J = 3.2 Hz, 8H, 4x H-4, 2x CONH-CH2-CH2-), 4.00 (t, J = 6.3 Hz, 4H, 4x H'-5), 3.97 – 3.88 (m, 12H, 4x H-3, 4x H'-3, 4x H'4), 3.88 – 3.78 (m, 12H, 4x H'-6, 4x H'-2), 3.68 (d, J = 6.4 Hz, 8H, 4x H-6), 3.64 (s, 4H, 2x CONH-CH2-CH2-). 13C NMR (151 MHz, Deuterium Oxide, extracted from HSQC) δ 125.03 (triazole), 108.89 (2xCHarom-2,6, 104.45 1x CHarom-4), 106.57 (4xCHarom-2',6'), 105.32 (2x CHarom-4'), 88.11(C-1), 100.53 (C'-1), 61.07 (triazole-CH2-), 70.93 (C-5), 69.65 (C-2), 77.35, 66.73, 78.13 (C'-5), 68.94, 72.72, 59.97, 68.70, 60.52 (C-6), 39.84 (CONH-CH2- CH2-). HR-ESI-TOF/MS (m/z): [M-H]- calcd. for C85H116N14O50, 2132.6964; found, 2132.6869

Compound 6. 1H NMR (600 MHz, Deuterium Oxide) δ 8.18 (s, 4H, 4H, triazole), 6.92 (s, 4H, 4xCHarom-2',6'), 6.56 (s, 2H, 2xC CHarom-4'), 5.54 (s, 4H, 4x H-1), 4.98 (s, 6H, 4x H'-1), 4.76 (s, 8H, 4x triazole-CH2-), 4.32 (s, 4H, 4x H-5), 4.25 (s, 4H, 4x H-2), 4.12 (s, 4H, 4x H-4), 4.01 – 3.49 (m, 32H, 4x H'-2, 4x H'-3, 4x H'-4, 4x H'-5, 4x H'-6, 4x H-3, 4x H-6), 3.19 (s, 4H, 2x CONH-CH2-), 1.42 (s, 4H, 2xCONH-CH2-CH2-), 1.05 (s, 16H, CONH-(CH2)12). HR-ESI-TOF/MS (m/z): [M-H]- calcd. for C86H128N14O46, 2092.8107; found, 2092.8028

Compound 8. hPG-azide (2.5 mg, 0.002 mmol of azide groups) was dissolved in water followed by the addition of ligand 7 (1.8 mg, 0.0032 mmol, 1.6 equiv). Copper sulphate pentahydrate (0.1 equiv) was dissolved in water separately and added to the reaction mixture. 0.3 equiv of sodium ascorbate was also dissolved in water separately and added to the reaction mixture. The reaction was carried out at 100 °C in the microwave for 1 h. Cuprisorb® resin was added to the reaction mixture and stirred to adsorb excess copper. The solvent was evaporated and the crude reaction mixture was purified by dialysis using a cellulose based dialysis cassette (MWCO: 2K) against deionized water for 3-4 days and freeze dried to get **8** in 80% yield as an off-white solid. The disappearance of the azide stretching peak in the IR spectra of the final compound confirmed that all of the azido polymer was consumed. 1H NMR (600 MHz, Deuterium Oxide) δ 8.03 (s, triazole), 5.18 – 3.16 (m, CH2 and CH, hPG-OH backbone; GB3), 2.31 – 2.18 (m, GB3; CH3), 0.85 (s, hPG core, CH2).

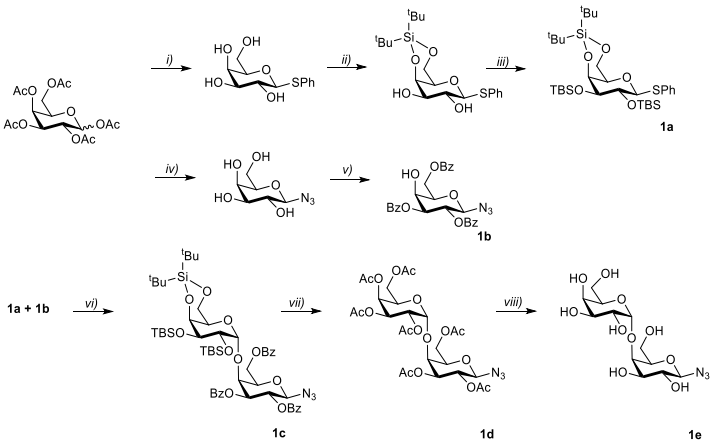
Compound 9. hPG-propargyl (5 mg, 0.0095 mmol of propargyl groups) was dissolved in water followed by the addition of the **1d** (8.2 mg, 0.012 mmol, 1.3 equiv) which was dissolved in DMF. Copper sulphate pentahydrate (0.1 equiv) was dissolved in water separately and added to the reaction mixture. 0.3 equiv of sodium ascorbate was also dissolved in water separately and added to the reaction mixture. The reaction was carried out at 80 °C in the microwave for 60 min. Cuprisorb® resin was added to the reaction mixture and stirred to adsorb excess copper. The crude mixture was extracted using ethyl

acetate and water. The protected polymer conjugate was then subjected to deacetylation using the standard procedure described above. The solvent was evaporated and the crude reaction mixture was purified by dialysis using a cellulose based dialysis cassette (MWCO: 2K) against deionized water for 3-4 days and freeze dried. The final product **9** was obtained in 75% yield as a white solid. The disappearance of the C≡CH stretching peak in the IR spectra of the final compound confirmed that all of the polymer was consumed. 1H NMR (600 MHz, Deuterium Oxide) δ 8.30 (s, triazole), 5.69 (d, J = 9.2 Hz, galabiose; H-1), 5.00 (d, J = 3.9 Hz, galabiose; H'-1), 4.51 – 3.30 (m, CH2 and CH, hPG-OH backbone; galabiose; H-2, H-3, H-4, H-5, H-6, H'-2, H'-3, H'-4, H'-5, H'-6), 1.25 (s, CH2 core), 0.84 (s, CH3 core).

3 Results

For the synthesis of the monovalent galabiose reagent, galactose pentaacetate was used as the common precursor for the synthesis of the glycosyl donor and acceptor (Scheme 1). Glycosyl donor **1a** was synthesized in three steps by thioglycoside preparation as the first step followed by silyl-protection of the sugar. Glycosyl donor **1b** was synthesized by azidation using trimethylsilyl-azide and benzoyl protection. Trifluoromethanesulfonic anhydride-mediated glycosylation afforded the disaccharide **1c** in moderate yields. Deprotection was performed over two steps without purification followed by acetylation to obtain **1d** which was used for conjugation with various dendrimers.

Scheme 1. Synthesis of galabiose azide [a]



[a] *Reagents and conditions: i) HSPh, BF3.Et2O, DCM, r.t., 16 h, 90%; NaOMe, MeOH, r.t. 90% ii) tBu2Si(OTf)2, pyridine, DMF, -40°C, >90% iii) TBDMSOTf, DMAP, pyridine, r.t. 70% iv) TMSN3, SnCl4, DCM, 95% ; NaOMe, MeOH, r.t., 16 h, 100% v) BzCl, pyridine, DCM, -80°C, 2h, 50% vi) Tf2O, Ph2SO, TTBP, DCM, -60°C, 1 h, 72% vii)) NaOMe, MeOH; HF, pyridine; Ac2O, pyridine, 63% viii) NaOH, MeOH, 90%.*

Building block 3,5-dihydroxybenzoic acid was used as the starting material for the synthesis of all four dendrimers (Figure 1). **2a** and **2c** were synthesized using previously reported procedures (29,30). Divalent **2b** was conveniently prepared by coupling methyl 3,5-bis(2-aminoethoxy)benzoate to propargyl chloroformate and was obtained in 88% yield. Amide coupling of **2a** to dodecane-1,12-diamine using BOP gave tetravalent dendrimer **2d** in 60% yield. Dendrimers (**2a, 2b, 2c, 2d**) were conjugated to **1d** by CuAAC, and deprotected to obtain final compounds **3, 4, 5** and **6** (Figure 3) in good yields.

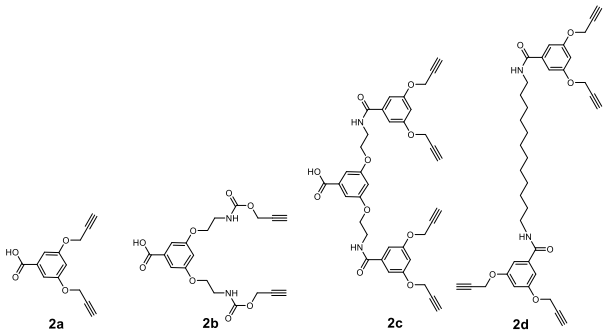
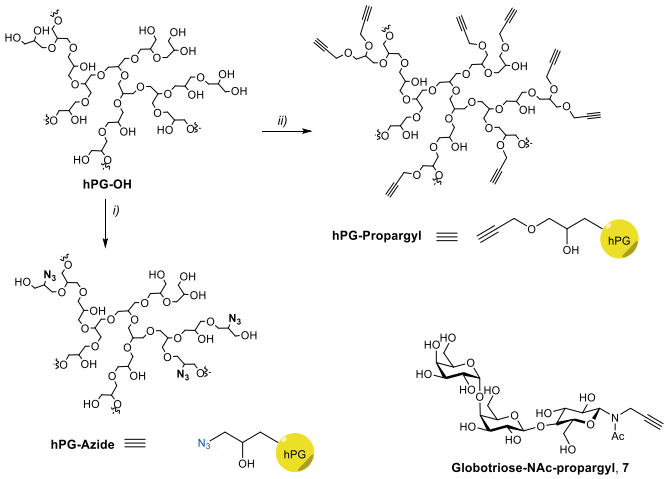


Figure 1. Di- and Tetravalent Dendrimers.

Glycidol, a reactive hydroxy-epoxide was used as an AB2 monomer and polymerization was initiated using tris(hydroxymethyl)propane (TMP). TMP was partially deprotonated and used as an initiator for the anionic polymerization carried out by slow monomer addition and yielding hPG-OH of ca. 9.4 kDa with 125 OH end groups, calculated using inverse-gated carbon and proton NMR (31). Azidation of the hPG was performed in two steps by first substituting the hydroxy groups of the hPG with the more reactive mesyl groups followed by azide substitution using sodium azide (32). Mesyl substitution of the hPG was calculated at 8% (ca. 10 mesyl end groups per molecule) using proton NMR and complete substitution with azide groups was confirmed by the absence of the mesyl protons (1H NMR) and the appearance of the azide stretching in the infrared spectra (IR) at 2110 cm-1. Propargylation of hPG was performed in a single step using propargyl bromide in 72% yield (33). The polymer was calculated to be 16% functionalized, which means ca. 20 propargyl end groups per molecule based on proton NMR and the IR spectra further confirmed this via the 2110 cm-1 peak.

hPG azide was conjugated by CuAAC to globotriose-NAc-propargyl (7, Scheme 2) to obtain **8** (Figure 3) in 80% yield. Similarly, conjugation of **1d** to hPG-propargyl following deprotection yielded final compound **9** (Figure 3) in 75% yield over two steps. Final polymers **8** and **9** were characterized by 1H-NMR and also by IR to check for the absence of the azide and alkyne stretching peaks respectively.

Scheme 2. Synthesis of Hyperbranched polymers [a]



[a] *Reagents and conditions: i) MsCl, TEA, DMF, 0°C- r.t., 16 h, 88%; NaN3, DMF, 60°C, quant. ii) NaH, KI, Propargyl bromide, DMF, 0°C- r.t., 72%*

Previously, inhibitors were tested for inhibition in ELISA assays using immobilization of the B subunit of Stx1 (Stx1B).24 In contrast we used an assay in which FSL-Gb3 was immobilized instead of the toxin (Supplementary figure 2), as this was deemed more realistic since in vivo the toxin is also free to move. FSL-GB3 is comprised of a functional component (F) which is GB3, conjugated via an O(CH2)3NH spacer (S) to an activated adipate derivative of dioleoylphosphatidylethanolamine (L). Monovalent **1e** was used as the reference in the ELISA and as expected showed millimolar inhibition of the toxin with an IC50 of approx. 5 mM. Divalent **3** and **4** also inhibited the toxin in the millimolar range (1 mM and 1.2 mM respectively) (**Table 1**). Clearly, the small variation in spacer length between dendrimer **2a** and **2b** did not cause any significant variation in potency. It was anticipated that if the divalent ligands bridge between sites 1 and 2 on a single toxin subunit (6), this would be more easily possible with the longer spacer of **4**. A stronger enhancement of the inhibition was observed with the tetravalent compounds **5** and **6** as both showed micromolar inhibition (20 µM and 13 µM respectively). Here again, the toxin did not discriminate between the elongated and more flexible dendrimer **2d** backbone with respect to **2c** (Figure 2).

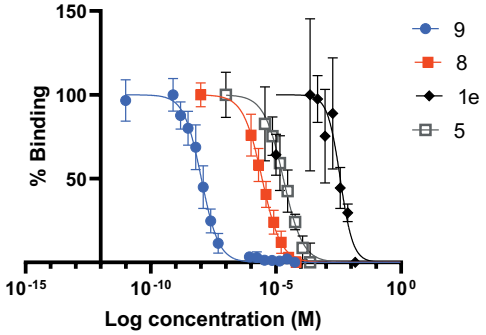


Figure 2. Inhibition of STxB1B (0.1 µg/mL) binding to a GB3 covered surface by compounds from left to right, 9 (blue), 8 (red), 1e (black), 5 (white).

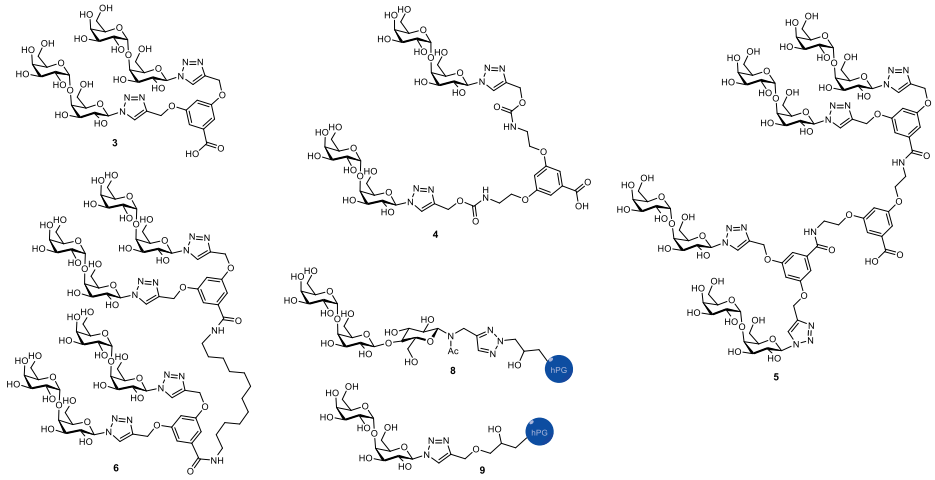


Figure 3. Dendrimeric- and Polymeric-Galabiose Conjugates

Table 1. Results of inhibition in Stx1B ELISA assay [a]

entry	construct	ligand	Valency (% functionalization of polymer)	IC_{50} (mM)	rel.pot.[b]	rel. pot. per sugar [c]
1	**1e**	galabiose	1	4968 ±1232	4968	1
2	**3**	galabiose	2	1070 ± 283	4.6	2.3
3	**4**	galabiose	2	1245 ±169	4	2
4	**5**	galabiose	4	19.9 ±2.4	250	62.5
5	**6**	galabiose	4	13.5 ±2.6	367	92
6	**8**	globotriose	10 (8%)	2.8 ± 0.2	1,774	187
7	**9**	galabiose	20 (16%)	0.0083 ±0.0006	598,554	29,928

[a]*Determined in an ELISA-like assay with Stx1B (0.1 mg/mL) and wells coated with Gb3,* [b]*Relative to the potency of galabiose for 3, 4, 5, 6 and 9 cRelative potency divided by the valency.*

We expected both compounds to bridge between the strongest of the three binding sites per subunit, the so-called site 2 (34), of the same pentamer separated by ca. 30 Å (6). For the decavalent hPG-Gb3 polymeric inhibitor **8**, low micromolar inhibition was seen (IC50 = 3 μM). Indeed the compound was more potent than the tetravalent **5** and **6** but not by much and the inherently stronger trisaccharide ligand it contains, could easily be responsible for this difference. Gratifyingly, the more highly substituted hPG-galabiose conjugate **9** was much more potent with an IC50 of 8 nM and a relative potency per sugar of ca. 30,000. These data make it the first nanomolar Stx inhibitor based on the disaccharide galabiose to the best of our knowledge.

A number of natural or synthesized oligosaccharides were subsequently tested for activity at the maximal non-toxic concentration of 2% (Figure 4) (35,36).Chitosan oligosaccharide (COS) is a cationic polymer obtained from crustaceans and consists of glucosamine repeating units and has several promising applications (37). COS (degree of acetylation: ≥95%) showed a 71% inhibition of the Stx1B with inhibitory effects seen as low as a 0.5% COS concentration (Figure 5). Alginate oligosaccharide (AOS), another naturally occurring polyuronic saccharide, is composed of β-D-mannuronic acid and α-l-guluronic acid (38). AOS have, among others, anti-tumor, anti-oxidative, immunoregulatory, anti-inflammatory activity (39). AOS did show 51% inhibition at a concentration of 0.5%. Curiously, higher AOS concentrations reduced the inhibition. Fructose and Galactose oligosaccharide (FOS and GOS) did not inhibit the toxin. Lactose was used as a negative control and did not show any activity (check the supplementary data).

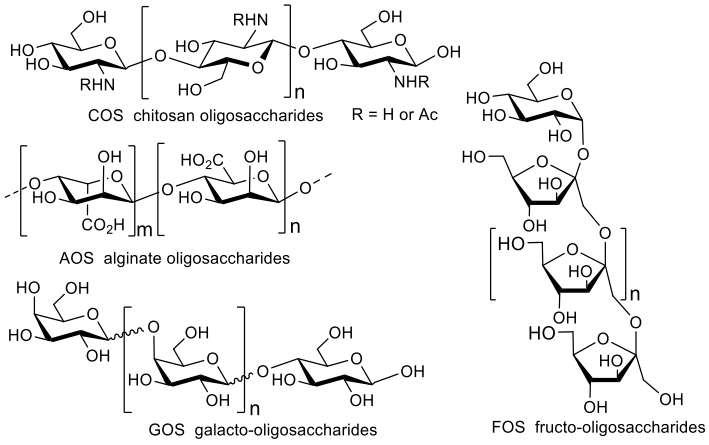


Figure 4. Structures of natural and synthetic inhibitors of Stx.

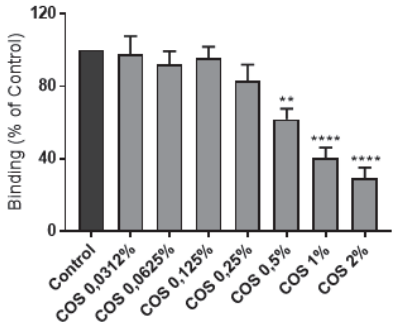


Figure 5 Stx1B binding inhibition by COS.

In order to evaluate the potential toxicity of the most effective Stx inhibitor, polymer **9**, toxicity tests were undertaken. Different concentrations of glycopolymer **9** (1, 10 and 100 nM) did not impair T84 cell viability after 24 h exposure as indicated by the MTT assay, while 10% ethanol (positive control) significantly reduced the cell viability (Figure 6a). Furthermore, as depicted in Figure 6b, 1, 10 and 100 nM of glycopolymer **9** did not significantly alter the TEER values compared to untreated cells after 24 h, whereas 10% ethanol strongly decreased the TEER values. TEER values (transepithelial electrical resistance) indicate the barrier integrity of epithelial cells.

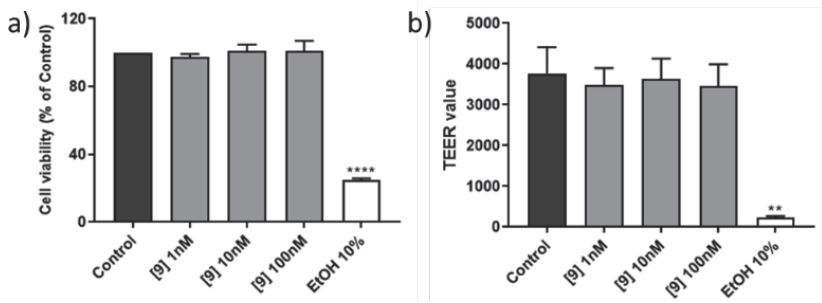


Figure 6. The effect of Glycopolymer 9 on intestinal cell viability and integrity. a) T84 cells grown on 96- well plates were exposed to 1, 10 and 100 nM glycopolymer 9 or 10% ethanol (positive control) for 24h, and cell viability was measured by a MTT reduction assay. The MTT values were presented as percentage MTT released by non-treated T84 cells as mean ± SEM of three independent experiments each performed in triplicate. b)T84 cells grown on transwell inserts were exposed to 1, 10 and 100 nM glycopolymer 9 or 10% ethanol (positive control) for 24h and TEER was measured as described in material and methods. The TEER values were presented as mean (Ω · cm2) ± SEM of three independent experiments each performed in triplicate. ** = P<0.001 compared to control. **** = P<0.0001 compared to control)

4 Discussion and conclusion

A growing number of Shigella infections contain the deadly Shiga toxin and the related STEC is also still a major threat without a proper therapeutic approach. In this study we aimed for a simple and effective toxin inhibitor by comparing three classes of carbohydrate-based inhibitors: glycodendrimers, glycopolymers and oligosaccharides. The glycodendrimers needed at least a tetravalent ligand to reach significant inhibition. One reason could be that it requires the bridging (40) between the two highest affinity sites (sites 2) of neighboring toxin subunits for a significant inhibitory effect. The smaller divalent compounds were too short to bridge the ca. 30 Å. It is likely that in addition to the chelation binding mode also aggregation of the toxin is taking place, as previously noted (41), and also for the related cholera toxin (42,43). Of the two glycopolymers it was striking that the more highly functionalized **9** was much more potent than **8**, despite having the weaker galabiose ligand. Clearly the high density of binding sites helps the inhibition as seen for the related cholera toxin inhibition with similar polymers (44,45), however with three binding sites per subunit, i.e 15 in total, the effects are more dramatic than for the cholera toxin with one binding site per subunit. Prior work, both theoretical and practical involving the Shiga-like toxin has clearly indicated that avidity effects like seen here are caused by intrinsic inter and intramolecular recognition events, but that on top of that there is an important combinatorial factor that describes the probabilities of binding events. This factor is very important and favorable and was shown to increase rapidly for higher valency systems, provided that the geometry of the multivalent ligand is appropriate for the target. In the case at hand, the particle like nature of the polymer is particularly suitable for toxins in comparison with other polymers. Furthermore, the polymers were both ca. 10 kDa but the ligand density is vastly different (valencies of 10 vs 20 for **8** and **9**). Clearly the statistical possibilities for the higher ligand density **9** are far greater and can overcome the lower intrinsic binding potency of the disaccharide vs the trisaccharide ligand.

Shigella spp. are highly infective bacteria. Only 10-100 microbes are already enough to cause infection that could become fatal, especially when it produces the toxin, as is also the case for STEC. The initial diarrhea followed by the toxin moving into the circulation provides a challenge for therapy. It takes ca. 5-9 days between the initial gastroenteritis until HUS occurs (46). In this time window a GI-based agent e.g. a food grade polysaccharide such as COS can be beneficial. This is true also as a preventative, in case of an outbreak as can happen with Shigella. In order to prevent the systemic diseases, i.e. HUS, a soluble non-toxic multivalent glycan with sufficient potency will likely be helpful. As such a further optimized dendrimer or the glycopolymer **9** based on hPG can be used. hPGs can be prepared on a large scale in an economical manner and have also been used in circulation (47). The utility of hPG is also well established in terms of safety and biocompatibility. We have previously also used hPG backbone to target cholera toxin and the flu virus with good results (44,48).

Notes

Galabiose azide synthesis was performed by Torben Heise. Stx1B expression, purification and the Stx1B assay conditions were optimized by Jie Shi. TEER measurement and the MTT assay were performed by Mostafa Asadpoor.

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Author Contributions

The manuscript was written through contributions of all authors. All authors approved of the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

References

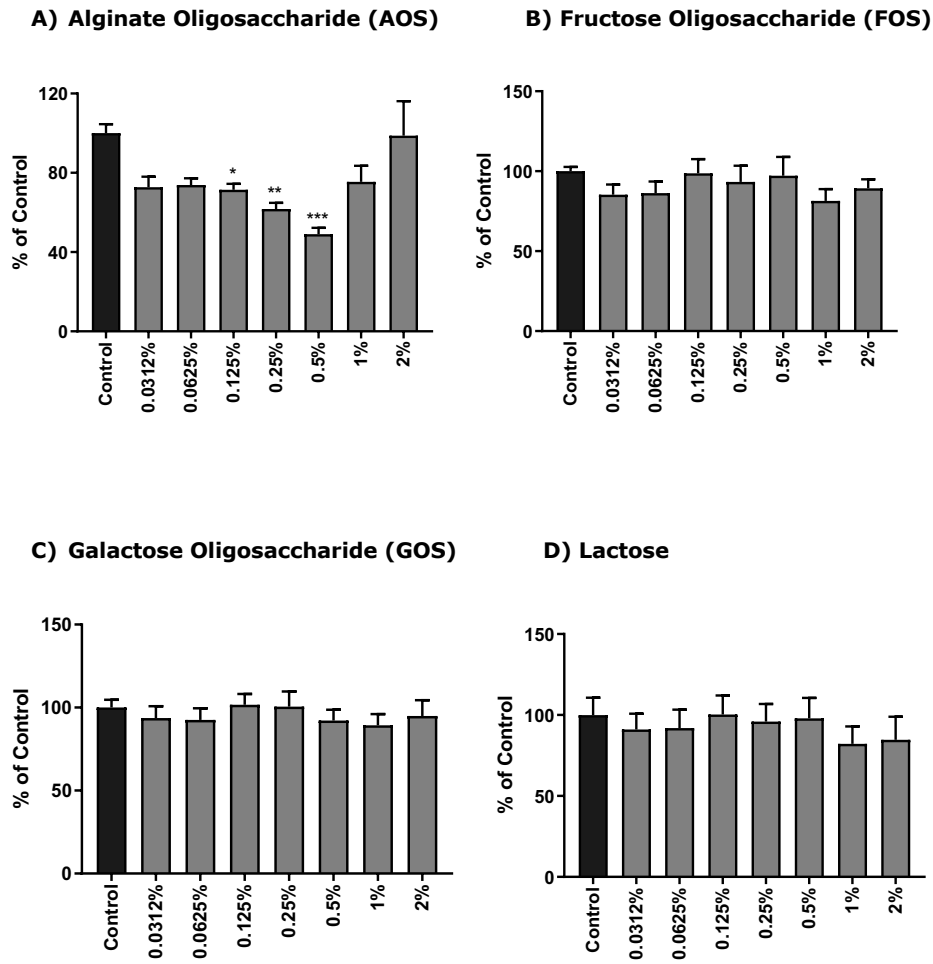
1. Liu J, Platts-Mills JA, Juma J, Kabir F, Nkeze J, Okoi C, et al. Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. Lancet. 2016;388(10051):1291–301.
2. Tesh VL, Burris JA, Owens JW, Gordon VM, Wadolkowski EA, O'Brien AD, et al. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. Infect Immun. 1993;61(8):3392–402.
3. Muniesa M, Hammerl JA, Hertwig S, Appel B, Brüssow H. Shiga Toxin-Producing Escherichia coli O104:H4: a New Challenge for Microbiology. Appl Environ Microbiol. 2012 Jun;78(12):4065–73.
4. Lee M-S, Koo S, Jeong DG, Tesh VL. Shiga Toxins as Multi-Functional Proteins: Induction of Host Cellular Stress Responses, Role in Pathogenesis and Therapeutic Applications. Toxins (Basel). 2016;8(3):1–23.
5. Bergan J, Dyve Lingelem AB, Simm R, Skotland T, Sandvig K. Shiga toxins. Toxicon. 2012;60(6):1085–107.
6. Ling H, Boodhoo A, Hazes B, Cummings MD, Armstrong GD, Brunton JL, et al. Structure of the Shiga-like toxin I B-pentamer complexed with an analogue of its receptor Gb3. Biochemistry. 1998;37(7):1777–88.
7. Lingwood C. Verotoxin Receptor-Based Pathology and Therapies. Front Cell Infect Microbiol. 2020;10(March):1–10.
8. Paletta ACC, Castro VS, Conte-Junior CA. Shiga Toxin-Producing and Enteroaggregative Escherichia coli in Animal, Foods, and Humans: Pathogenicity Mechanisms, Detection Methods, and Epidemiology. Vol. 77, Current Microbiology. 2020. p. 612–20.
9. Wong CS, Jelacic S, Hareeb RL, Watkins SL, Tarr PI. The Risk of the Hemolytic-Uremic Syndrome After Antibiotic Treatment of Eschericia Coli 0157:H7 Infections. N Engl J Med. 2000;342:1930–6.
10. Fogolari M, Mavian C, Angeletti S, Salemi M, Lampel KA, Maurelli AT. Distribution and characterization of Shiga toxin converting temperate phages carried by Shigella flexneri in Hispaniola. Infect Genet Evol. 2018;65(April):321–8.
11. Carter CC, Fierer J, Chiu WW, Looney DJ, Strain M, Mehta SR. A Novel Shiga Toxin 1a-Converting Bacteriophage of Shigella sonnei With Close Relationship to Shiga Toxin 2-Converting Pages of Escherichia coli. Open Forum Infect Dis. 2016 Apr;3(2):ofw079.
12. Nyholm O, Lienemann T, Halkilahti J, Mero S, Rimhanen-Finne R, Lehtinen V, et al. Characterization of Shigella sonnei Isolate Carrying Shiga Toxin 2–Producing Gene. Emerg Infect Dis. 2015;21(May):891–2.
13. Tóth I, Sváb D, Bálint B, Brown-Jaque M, Maróti G. Comparative analysis of the Shiga toxin converting bacteriophage first detected in Shigella sonnei. Infect Genet Evol. 2016;37(2016):150–7.
14. Gray MD, Lampel KA, Strockbine NA, Fernandez RE, Melton-Celsa AR, Maurelli AT. Clinical isolates of shiga toxin 1a-producing Shigella flexneri with an epidemiological link to recent travel to hispaniola. Emerg Infect Dis. 2014;20(10):1669–77.
15. Gupta SK, Strockbine N, Omondi M, Hise K, Fair MA, Mintz E. Short report: Emergence of Shiga toxin 1 genes within Shigella dysenteriae type 4 isolates from travelers returning from the Island of Hispa??ola. Am J Trop Med Hyg. 2007;76(6):1163–5.
16. Sváb D, Bálint B, Vásárhelyi B, Maróti G, Tóth I. Comparative Genomic and Phylogenetic Analysis of a Shiga Toxin Producing Shigella sonnei (STSS) Strain. Front Cell Infect Microbiol. 2017;7(May):1–10.
17. Bekal S, Pilon PA, Cloutier N, Doualla-bell F, Longtin J. Identification of Shigella flexneri isolates carrying the Shiga toxin 1-producing gene in Quebec , Canada , linked to travel to Haiti. Can J Microbiol. 2015;61(September):995–6.
18. Beutin L, Strauch E, Fischer I. Isolation of Shigella sonnei l y s o g e n i c for a bacteriophage encoding gene for production of Shiga toxin. Lancet. 1999;353:1498.
19. Lamba K, Nelson JA, Kimura AC, Poe A, Collins J, Kao AS, et al. Shiga Toxin 1 – Producing Shigella sonnei Infections, California, United States, 2014–2015. Emerg Infect Dis. 2016;22:679–86.

20. Watanabe M, Igai K, Matsuoka K, Watanabe T, Yanoshita R, Terunuma D, et al. Structural Analysis of the Interaction between Shiga Toxin B Subunits and Linear Polymers Bearing Clustered Globotriose Residues Structural Analysis of the Interaction between Shiga Toxin B Subunits and Linear Polymers Bearing Clustered Globotriose Residu. Infect Immun. 2006;74(3):1984–8.
21. Takeda T, Yoshino KI, Adachi E, Sato Y, Yamagata K. In vitro assessment of a chemically synthesized Shiga toxin receptor analog attached to Chromosorb P (Synsorb Pk) as a specific absorbing agent of Shiga toxin 1 and 2. Microbiol Immunol. 1999;43(4):331–7.
22. Trachtman H, Cnaan A, Christen E, Gibbs K, Zhao S, DWK A, et al. Effect of an oral Shiga toxin-binding agent on diarrhea-associated hemolytic uremic syndrome in children: a randomized controlled trial. JAMA J Am Med Assoc. 2003;290(10):1337–44.
23. Ganji A, Islami M, Ejtehadifar M, Zarei-Mehrvarz E, Darvish M. Nanobody and aptamer as targeting moiety against bacterial toxins: Therapeutic and diagnostic applications. Rev Med Microbiol. 2019;30(3):183–90.
24. Kitov PI, Sadowska JM, Mulvey G, Armstrong GD, Ling H, Pannu NS, et al. Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. Nature. 2000;403(6770):669–72.
25. Mulvey GL, Marcato P, Kitov PI, Sadowska J, Bundle DR, Armstrong GD. Assessment in mice of the therapeutic potential of tailored, multivalent Shiga toxin carbohydrate ligands. J Infect Dis. 2003;187(4):640–9.
26. Nishikawa K, Matsuoka K, Kita E, Okabe N, Mizuguchi M, Hino K, et al. A therapeutic agent with oriented carbohydrates for treatment of infections by Shiga toxin-producing Escherichia coli O157:H7. Proc Natl Acad Sci U S A. 2002;99(11):7669–74.
27. Calderón M, Quadir MA, Sharma SK, Haag R. Dendritic polyglycerols for biomedical applications. Adv Mater. 2010;22:190–218.
28. Asadpoor M, Peeters C, Henricks PAJ, Varasteh S, Pieters RJ, Folkerts G, et al. Anti-pathogenic functions of non-digestible oligosaccharides in vitro. Nutrients. 2020 Jun;12(6):1789.
29. Rijkers DTS, van Esse GW, Merkx R, Brouwer AJ, Jacobs HJF, Pieters RJ, et al. Efficient microwave-assisted synthesis of multivalent dendrimeric peptides using cycloaddition reaction (click) chemistry. Chem Commun. 2005 Sep;13(36):4581–3.
30. Dijkgraaf I, Rijnders AY, Soede A, Dechesne AC, Van Esse GW, Brouwer AJ, et al. Synthesis of DOTA-conjugated multivalent cyclic-RGD peptide dendrimers via 1,3-dipolar cycloaddition and their biological evaluation: Implications for tumor targeting and tumor imaging purposes. Org Biomol Chem. 2007;5(6):935–44.
31. Sunder A, Hanselmann R, Frey H, Mülhaupt R. Controlled synthesis of hyperbranched polyglycerols by ring-opening multibranching polymerization. Macromolecules. 1999;32(13):4240–6.
32. Bhatia S, Lauster D, Bardua M, Ludwig K, Angioletti-Uberti S, Popp N, et al. Linear polysialoside outperforms dendritic analogs for inhibition of influenza virus infection in vitro and in vivo. Biomaterials. 2017;138:22–34.
33. Kurniasih IN, Liang H, Möschwitzer VD, Quadir MA, Radowski M, Rabe JP, et al. Synthesis and transport properties of new dendritic core-shell architectures based on hyperbranched polyglycerol with biphenyl-PEG shells. New J Chem. 2012;36(2):371–9.
34. Shimizu H, Field RA, Homans SW, Donohue-Rolfe A. Solution structure of the complex between the B-subunit homopentamer of verotoxin VT-1 from Escherichia coli and the trisaccharide moiety of globotriaosylceramide. Biochemistry. 1998;37(31):11078–82.
35. Akbari P, Braber S, Alizadeh A, Verheijden KAT, Schoterman MHC, Kraneveld AD, et al. Galacto-oligosaccharides protect the intestinal barrier by maintaining the tight junction network and modulating the inflammatory responses after a challenge with the mycotoxin deoxynivalenol in human caco-2 cell. J Nutr. 2015;145(7):1604–13.
36. Varasteh S, Braber S, Garssen J, Fink-Gremmels J. Galacto-oligosaccharides exert a protective effect against heat stress in a Caco-2 cell model. J Funct Foods. 2015;16:265–77.
37. Naveed M, Phil L, Sohail M, Hasnat M, Baig MMFA, Ihsan AU, et al. Chitosan oligosaccharide (COS): An overview. Int J Biol Macromol. 2019;129:827–43.

38. Liu J, Yang S, Li X, Yan Q, Reaney MJT, Jiang Z. Alginate Oligosaccharides: Production, Biological Activities, and Potential Applications. Compr Rev Food Sci Food Saf. 2019 Nov;18(6):1859–81.
39. Xing M, Qi C, Yu W, Han X, Jiarui Z, Qing Z, et al. Advances in research on the bioactivity of alginate oligosaccharides. Mar Drugs. 2020;18(3).
40. Wittmann V, Pieters RJ. Bridging lectin binding sites by multivalent carbohydrates. Chem Soc Rev. 2013 May;42(10):4492–503.
41. Hilaire PMS, Boyd MK, Toone EJ. Interaction of the Shiga-like Toxin Type 1 B-Subunit with Its Carbohydrate Receptor. Biochemistry. 1994;33(48):14452–63.
42. Fu O, Pukin A V., van Ufford HCQ, Branson TR, Thies-Weesie DME, Turnbull WB, et al. Tetra- versus Pentavalent Inhibitors of Cholera Toxin. Chem Open. 2015;4:471–7.
43. Ommen DDZ, Pukin A V, Fu O, Quarles LHC, Van U, Janssens HM, et al. Functional Characterization of Cholera Toxin Inhibitors Using Human Intestinal Organoids. J Med Chem. 2016;59:6968–72.
44. Haksar D, De Poel E, Van Ufford LQ, Bhatia S, Haag R, Beekman J, et al. Strong Inhibition of Cholera Toxin B Subunit by Affordable, Polymer-Based Multivalent Inhibitors. Bioconjug Chem. 2019;30(3):785–92.
45. Haksar D, Quarles Van Ufford L, Pieters RJ. A hybrid polymer to target blood group dependence of cholera toxin. Org Biomol Chem. 2019;18(1):52–5.
46. Armstrong GD, Rowe PC, Goodyer P, Orrbine E, Terry P, Wells G, et al. A Phase I Study of Chemically Synthesized Verotoxin (Shiga-like Toxin) Pk-Trisaccharide Receptors Attached to Chromosorb for Preventing Hemolytic-Uremic Syndrome. J Infect Dis. 1995;171:1042–5.
47. Chapanian R, Constantinescu I, Brooks DE, Scott MD, Kizhakkedathu JN. In vivo circulation, clearance, and biodistribution of polyglycerol grafted functional red blood cells. Biomaterials. 2012;33(10):3047–57.
48. Lu W, Du W, Somovilla VJ, Yu G, Haksar D, de Vries E, et al. Enhanced Inhibition of Influenza A Virus Adhesion by Di- and Trivalent Hemagglutinin Inhibitors. J Med Chem. 2019;62:6398–404.
49. Shin IS, Nishikawa K, Maruyama H, Ishii S. Histidine-tagged Shiga toxin B subunit binding assay: Simple and specific determination of Gb3 content in mammalian cells. Chem Pharm Bull. 2006;54(4):522–7.
50. Novoa A, Machida T, Barluenga S, Imberty A, Winssinger N. PNA-Encoded Synthesis (PES) of a 10 000-Member Hetero-Glycoconjugate Library and Microarray Analysis of Diverse Lectins. Chembiochem. 2014 Sep;15(14):2058–65.
51. Ohlsson J, Larsson A, Haataja S, Alajaaski J, Stenlund P, Pinkner JS, et al. Structure–activity relationships of galabioside derivatives as inhibitors of. Org Biomol Chem. 2005;3:886–900.

Supplementary figures

Stx 1B Inhibition Graphs



Supplementary figures Stx1B binding inhibition to Gb3 receptors by AOS (A), FOS (B), GOS (C), and Lactose (D).

Chapter 8

General Discussion

Bacteria such as *Escherichia coli, Streptococcus agalactiae, Staphylococcus aureus* and *Clostridioides difficile* are well recognized for causing significant infections in humans, which can lead to major complications such as diarrhea, meningitis and hemolytic uremic syndrome. These pathogenic bacteria can have disastrous consequences for immunocompromised people, children and elderly. The more effective a pathogen is during colonization and invasion, the more likely a bacterial infection will develop (1). Bacterial adhesion occurs before onset of colonization and is an important factor in bacterial pathogenesis. Bacteria tend to use adherence factors, like fimbriae, to interact with cell surfaces. Adherence factors often bind to specific binding sights on the surface of target cells. For instance, membrane glycoconjugates, which commonly include N-acetylneuraminic acid, are responsible for S-fimbriae adhesion to the host cell surface (2).

Various colonizing bacteria have the ability to form well-organized biofilm communities. Biofilm formation is one of the common immune evasion mechanisms. Biofilm formation is a smart strategy of bacteria to protect themselves from antimicrobial agents and the immune system (3). When the host defense system and the antimicrobial agents are ineffective in preventing the bacterial growth in the biofilm, the infection can spread to other parts of the body (4). Successful invasion depends on bacterial pathogen growth and proliferation. To further improve the chances of successful invasion, pathogens express and use a variety of virulence factors, which elicit damage to host cells. Toxins are important virulence factors of bacterial pathogens that disturb ion channel functionality and barrier integrity, and may cause direct damage to host tissues, and this allows the pathogens to spread and accumulate throughout the body (5). The induction of inflammation is how eukaryotes respond to an infection. This is a protective reaction of cells to pathogens or their toxins. The inflammatory cascade is coordinated by communication between different immune cells and molecular signals (6).

There is a wide range of antibiotics available to combat bacterial infections. However, resistance has evolved for almost every antimicrobial drug, presenting a serious global health problem and an urgent need to develop alternative treatments to conventional antibiotics (7). Non-digestible oligosaccharides (NDOs) could be a potential alternative. NDOs been found to directly inhibit bacterial growth or even kill bacteria, and may even increase the efficacy of antibiotics. NDOs have received increasing attention in recent years due to their multiple health-beneficial characteristics (8,9). NDOs are chemically stable compounds and are well-established dietary fibers and prebiotics (10). NDOs not only exhibit microbiota-dependent properties (described in **Chapter 5**), but also seem to be crucial inhibitors to treat human pathogenic infections via microbiota-independent mechanisms (described in **Chapter 2** and **5**). Based on the type and structural characteristics, NDOs can exert different anti-pathogenic effects. NDOs can directly inhibit pathogenicity of various pathogens by reducing bacterial adherence and growth, or biofilm formation (**Chapter 3**, **4**, **6**) (11). In addition, NDOs have anti-inflammatory properties and can promote epithelial barrier functionality by several pathways including regulating tight

junction (TJ) abundance and dynamics (**Chapter 3** and **6**) (12). Although there is some evidence of the antipathogenic capabilities of NDOs, less is known about the antimicrobial mechanisms of NDOs (for instance of *E. coli, S. agalactia,* and *S. aureus)*, their structure-function relationships and their species- and strain dependency and the effectiveness of combination therapy: NDOs combined with antibiotics.

This thesis investigated the effects of NDOs (alginate-oligosaccharides (AOS), chitosan-oligosaccharides (COS), fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS)) on the defense against several common bacterial pathogens (*E. coli, S. agalactia, S. aureus,* and *C. difficile*) in different steps of the pathogenesis: bacterial growth, bacterial adhesion, biofilm formation, inflammation and bacterial toxin activity. The knowledge about the antimicrobial strategies of NDOs was used to further investigate combination therapy by combining NDO treatment with antibiotics to increase bacterial sensitivity to specific antibiotics.

In this thesis the anti-microbial functionality of NDOs against *E. coli, S. agalactia, S. aureus, and C. difficile* was studied via 5 different ways:

Bacterial growth inhibition

Our findings showed that AOS can selectively inhibit bacterial growth. AOS inhibited growth of *E. coli* (up to 30%) at a concentration of 0.5%, and growth inhibition of *C. difficile* (up to 80%) was observed at a concentration of 8%. Importantly, the growth of *S. agalactia* was significantly inhibited by AOS from a concentration of 2% to a maximum inhibition of 81% at an AOS concentration of 8%. AOS did not exert any significant reduction on the growth of *S. aureus*. In contrast, Hu *et al.* demonstrated a higher growth inhibitory activity of a depolymerized product of alginate against another strain of S*. aureus* (13). These differences in the anti-pathogenic activities of AOS might be linked to the chemical interaction between the structural features of AOS and variations in the targeted structures of the different bacterial strains. In summary, the anti-growth activity of AOS might be concentration-dependent and strain-dependent. One probable explanation of such an effect is attributed to the anionic nature of AOS. Craft *et al.* demonstrated that sialylated human milk oligosaccharides (HMOs), which are negatively charged, exert significant antimicrobial activity against *S. agalactia* (14). However, neutral fucosylated HMOs did not have any substantial effects on microbial pathogens (15). Hence, the negative charge of AOS is thought to play an important role in their antimicrobial activities.

On the other hand, COS increased the growth of *S. agalactia, S. aureus,* and *E.coli* and were ineffective on the growth of *C. difficile*. Due to the increase in bacterial growth, it is hypothesized that *S. agalactia, S. aureus,* and *E.coli* utilized COS as a metabolic source. Similar results were found in an earlier study, in which chitosan stimulated *S. agalactia* growth (16). These observations were contrary to previous reports finding that COS had significant antimicrobial activities via the interaction between the positively charged groups of COS and the negatively charged bacterial membrane residues (e.g., lipids,

proteins, and carbohydrate) (17–21). The antibacterial effect of COS significantly increased as the degree of polymerization or/and molecular weight (MW) increased (22). Jeon *et al.* showed that COS with a higher molecular weight (MW ≥10 kDa) were more effective in the inhibition of different microorganisms compared to fractions with a lower MW (17). The experimental and methodological differences between studies cannot be ruled out, such as distinct structural characteristics of COS and various bacterial strains.

Furthermore, in our studies, FOS and GOS did not inhibit *E. coli* growth (except at very high GOS concentrations, which might be attributed to changes in osmolarity). In addition, no evidence regarding the inhibitory effects of FOS and GOS on growth of *S. aureus, S. agalactia* and *C. difficile* has been reported so far (11). Unlike AOS and COS, FOS and GOS lack functional groups capable of bearing a charge. Therefore, AOS and COS, but not FOS and GOS, might be involved in ionic interaction, which results in the anti-pathogenic effects.

Although all NDOs feature a comparable carbohydrate structure, they have quite different physicochemical characteristics that determine their interactions with surface receptors or other types of molecule-molecule interactions. Based on these differences, it can be hypothesized that the growth of pathogens, might be related to the ionic interaction between charged NDOs and the exterior surface of bacteria, and/or an altered ion flux. In conclusion, the anti-growth capacity of NDOs against pathogenic bacteria appears to be pathogen-, strain- and concentration-dependent.

Bacterial adhesion inhibition

AOS possess anti-adhesive properties against *E. coli*. However, COS, FOS and GOS did not display significant anti-adherence activity against the attachment of *E. coli* to intestinal epithelial cells. Similarly, Shoaf *et al.* (2006) found no evidence of an anti-adhesive effect of FOS (23). In contrast to our findings, different studies showed the anti-adhesive activities of GOS against *E. coli* (enteropathogenic strain) (11). Since the *E. coli* used in our study is not an enteropathogenic *E. coli* (EPEC), an explanation might be that the anti-adhesive properties of GOS against *E. coli* are strain-dependent. There is currently limited evidence for an anti-adhesive effect of COS. COS was shown to be an adhesion inhibitor of EPEC but ineffective against verotoxin-producing *E. coli* (VTEC). These results support our study in which COS did not inhibit the adhesion of non-EPEC *E.coli*. In contrast to a variety of studies focusing on the anti-adhesive activities of NDOs against *E.coli*, studies concerning the anti-adhesion functionalities of various NDOs (AOS, COS, FOS, GOS) against *Clostridium spp.*, *S. agalactia* and *S. aureus* are limited. To date, only FOS inhibited the adhesion of several *C. difficile* strains to human epithelial cells, possibly by impacting the bacterial surface proteins and adhesins (24). On the other hand, GOS exhibited significant anti-adhesive activities against several gram-negative bacteria such as *Cronobacter sakazakii*, *E.coli* and *Salmonella typhimurium* (11). Therefore, the anti-adhesion property of NDOs might be pathogen- and strain-dependent.

The mechanism of action for decreasing the adherence of bacterial pathogens to intestinal epithelial cells by NDOs still needs to be unraveled. There are two generally accepted explanations for the anti-adhesive properties of NDOs. First, NDOs (specifically charged-NDOs like AOS and COS) inhibit bacterial motility and swarming, causing bacteria to aggregate instead of spreading and colonizing (11). Second, NDOs (specifically non-charged NDOs like GOS and mannan-oligosaccharides (MOS)) can bind to the same structures as the pathogens which prevents pathogen adhesion to cell surface glycan receptors (5,11). For example, *Entamoeba histolytica* is a pathogen that uses β–galactose structures on intestinal epithelial cells for lectin-mediated adhesion and GOS have the same pattern (25).

Bacterial biofilm inhibition

AOS and COS are capable of inhibiting biofilm formation of *S. aureus*. The biofilm inhibitory effect is considered when at least 90% inhibition in biofilm formation is observed. AOS at a 16% concentration and COS at 8% and above were found to have an inhibitory effect on biofilm formation of *S. aureus*. Even at non-toxic concentrations (1 - 2%)(demonstrated in intestinal epithelial cell models), COS significantly decreased the biofilm formation of *S. aureus*. On the other hand, AOS and COS did not inhibit the biofilm formation of *S. agalactia* (except a slight reduction by 16% AOS which did not reach the 90% inhibitory threshold). The anti-biofilm properties of AOS and COS have been demonstrated against other bacterial strains like *Pseudomonas aeruginosa* (11), but there is limited research on their anti-biofilm activity against *E. coli* and *C. difficile*.

In general, biofilm formation is a bacterial adaptation strategy to environmental stressors like nutrient limitation, anoxia, and antimicrobial agents. Bacteria in a biofilm can alter their gene expression, metabolism and protein production to reach a lower metabolic rate. Biofilms are primarily composed of bacterial cells and extracellular polymeric substances (EPS). EPS are composed of multiple components, including proteins, DNA/RNA, carbohydrates, and water (26). The structure and composition of biofilms can differ between bacterial strains. To illustrate, polysaccharide intercellular adhesin (PIA) is the most important positively charged polysaccharide in the biofilm produced by Staphylococci (27). However, negatively-charged alginate is the most important matrix polysaccharide in *Pseudomonas* biofilms (11). It is hypothesized that the electrostatic interaction between the charged components of the biofilm matrix and the NDOs impacts the formation and maturation of a biofilm. The anti-biofilm effect of AOS against *S. aureus* is thought to be caused by the negative charge of AOS disrupting the intramolecular interactions in the EPS. In contrast, the anti-biofilm properties of COS are likely due to the polycationic nature of its protonated amino groups interacting electrostatically with the negatively charged biofilm components (e.g. proteins and eDNA) (27). In support of our findings, neutral NDOs like FOS and GOS have not been reported to exert anti-biofilm activity against *S. aureus*. Furthermore, no effects were observed when AOS and COS were added after the formation of the biofilm, potentially meaning that COS and AOS biofilm inhibitory effects impact the formation of biofilms rather than the destruction of already established biofilms (**Chapter**

4) (27). Another alternative pathway in the inhibition of biofilm formation of NDOs, like AOS and COS, could be related to the inhibition of pathogenic swarming and motility: two vital mediators in biofilm formation of gram negative bacteria like *E. coli* and *P. aeruginosa* (11). Interestingly, the motility inhibition of *P. aeruginosa* by anionic AOS might be caused by adhesion to the pathogenic exterior or flagella. Furthermore, the chelation of ions like Ca^{+2} by a negatively charged NDOs like AOS can result in inhibition of bacterial motility (28). Inhibited motility and the resulting cellular aggregation suppresses the formation and growth of biofilms (11). In conclusion, charged NDOs like AOS and COS can modulate biofilm formation and development, which might be due to the charge of the NDOs and/or the electrostatic interaction between NDOs and bacterial surface charges or ions.

Inflammation inhibition

A bacterial infection can cause inflammation. Pre-treatment of intestinal epithelial HT-29 cells with AOS, COS and GOS decreased the *E. coli*-induced pro-inflammatory cytokine (IL-8) response, but FOS did not exhibit a significant IL-8 reduction under the same conditions. The immunomodulatory properties of NDOs are not yet fully understood. The NDOs: GOS, COS and AOS are Toll-like receptors 4 (TLR4) ligands and could inhibit NF-B activation and MAPK phosphorylation in LPS-stimulated cells (29–31). Furthermore, we demonstrated in another study that both AOS and COS can reduce the inflammation provoked by a TLR2 ligand: *S. aureus* Lipoteichoic Acid (data not shown). Hence, a competition between NDOs and pathogenic bacteria such as *E. coli* and *S. aureus* for binding to TLRs (particularly TLR2 and TLR4) might be an explanation for the anti-inflammatory activity of some NDOs.

Alternatively, anti-inflammatory properties of AOS, COS and GOS might be contributed to their ability to prevent gut barrier disruption caused by bacterial infection. Enterotoxigenic *E. coli* is recognized to have a great impact on gut epithelial monolayer disruption. Camilleri (2019) showed that increased intestinal permeability causes acute intestinal inflammation (32). Furthermore, we demonstrated that both AOS and COS protect the epithelial barrier against an enterotoxin that usually causes enhanced permeability. GOS also have the ability to protect the intestinal epithelial barrier by preserving the TJ network (33). At least in part, NDOs may moderate the inflammatory response by limiting intestinal barrier damage.

Moreover, researchers found a relationship between the anti-inflammatory activity of NDOs and intestinal epithelium-derived galectin-9, since Kivit *et al* (2013) hypothesized that the immunomodulating effects of a GOS/FOS mixture involve intestinal epithelium-derived galectin-9 (34). Furthermore, Zenhom M *et al* (2011) suggested that anti-inflammatory effects of NDOs might be linked to the activation of the nuclear receptor (PPAR) which modulates the peptidoglycan recognition protein 3 (PGlyRP3) (35). Other suitable candidates for illustrating the immunomodulatory activity of NDOs might be binding to carbohydrate receptors, including Ca^{2+} dependent C type lectin receptors and Ca^{2+} independent C type lectin receptors, as outlined in the Y Cai *et al* review article (2020) (36). Further studies are needed to robustly test these hypotheses linked to the findings in this thesis.

Besides the direct effect of NDOs on certain immunological and intestinal epithelial cells, NDOs exhibit immunomodulatory properties which are likely mediated by beneficial gut bacteria and bacterial metabolites such as short chain fatty acids (5). Several beneficial bacteria, including *Bifidobacterium spp.*, have been found to increase the number of IgA-producing cells in the lamina propria, causing sIgA secretion in the luminal mucus layers and inhibiting bacterial colonization of the epithelium (37). Furthermore, beneficial bacteria can stimulate T-regulatory cells to produce and secrete anti-inflammatory cytokines, including IL-10 and TGF-β (38). The microbiota-dependent effects of NDOs on pathogenic bacteria were discussed in **Chapter 5** of this thesis. Although microbiota-dependent effects were not the major focus of our research projects, these findings should be taken into account in future *in vivo* studies.

Toxin inhibition

In **Chapter 6** of this thesis, we found that both AOS and COS significantly decreased *C. difficile* toxin A (TcdA)-induced cell death. 2% AOS and COS exhibited the strongest and most significant effects in reducing TcdA-induced Caco-2 cell cytotoxicity after 24 hours. Unlike AOS, COS already demonstrated a significant protective effect after 3 hours. TcdA causes cytotoxicity in cells by inducing programmed cell death. Both AOS and COS showed *in vitro* anti-apoptotic capabilities according to previous investigations on intestinal epithelial cells (5). In porcine jejunal (IPEC-2) monolayers, AOS were found to reduce TNF-induced and TLR-4/NF-B-mediated apoptosis (39,40). COS were also shown to prevent TNF-α-induced apoptosis in T84 intestinal epithelial cells (41). As a result, blocking apoptosis via both AOS and COS might be a potential protective mechanism against TcdA cytotoxicity.

Moreover, in this thesis we demonstrated that AOS and COS have protective capacities against TcdA-mediated intestinal barrier disruption. AOS alleviated and COS completely abolished the TcdA-induced transepithelial electrical resistance (TEER) drop. Both NDOs completely prevented the flux of lucifer yellow from the apical to the basolateral side of the transwell inserts. So far, we know that decreasing inflammation/cytokine levels (like IL-8, IL-6, and TNF-α), alleviating cytotoxicity and altering protein levels of two critical TJ proteins: ZO-1 and occludin, were not the likely mechanisms of NDOs action against TcdA. However, both AOS and COS accelerated the re-assembly of intestinal epithelial TJs, suggesting that they can reinforce the intestinal barrier by boosting the sealing of the paracellular route. The TJ re-assembly process depends on the activation of AMPK, a crucial TJ assembly/disassembly regulator (42), therefore COS and AOS seem to stimulate AMPK signaling. Another possibility is that TcdA impaired intestinal epithelial cell integrity by mislocalizing TJ proteins, which COS/AOS potentially regulate.

Receptor antagonism is another likely explanation for the barrier-protecting properties of AOS and COS as the carbohydrate structure of AOS and COS can bind to the TcdA cell surface domains such as carboxy-terminal host cell-binding domain (43). However, more research is required to confirm the actual mechanism of actions.

Interestingly, in **Chapter 7** of this thesis we demonstrated that AOS and COS are capable of inhibiting Shiga toxin B (Stx1B) activity. COS inhibited Stx1B by 71% at a concentration of 2%, whilst AOS inhibited Stx1B by 51% at a concentration of 0.5%. FOS and GOS did not exert any significant inhibition of Shiga toxin binding to Gb3 receptors. Shiga toxin producing *E. coli* (STEC) and *Shigella dysenteriae* cause a large range of acute illnesses by secreting shiga toxin. The life-threatening effects of Shiga toxin are related to the possible development of hemolytic-uremic syndrome (HUS) which can lead to renal failure (44). As a result of an infection with Shiga toxin-producing bacteria, children and elderly are at a higher risk of developing HUS. Unfortunately, there is no clinically recognized cure for Shiga toxin-producing bacteria and therapy for shiga toxins is only supportive. Treatment with common antibiotics is not recommended for Shiga-producing bacteria. When an antibiotic is administered, the bacteria produce more shiga toxin, increasing the risk of developing HUS (45). Since the binding sites on the Shiga toxin B subunit are critical for entering cells by endocytosis, blocking the binding sites on the Shiga toxin would prevent the harmful consequences. When there is no endocytosis, no pathogenic consequences, such as HUS arise. HMOs and pectin oligosaccharides (POS) have been demonstrated to bind to Shiga toxin, partly neutralizing it and attenuating its cytotoxicity (46,47). The ability of AOS and COS to partly prevent Shiga toxin binding to the Gb3 receptor was revealed in this thesis. In addition, our chemist collaborators synthesized Gb3 inhibitors, based on NDO structures, that completely blocked the binding of the Shiga toxin 1B subunit to Gb3 receptors. The inhibitory effect of AOS and COS on binding of the Shiga toxin to Gb3 shows the therapeutic potential of NDOs against Shiga toxin. This capability might help with the problem of STEC or *S. dysenteriae* infections that are still incurable. However, *in vivo* experiments are required to confirm the efficacy and safety of these compounds in “real-life” infections.

Effectivity of combination therapy: NDOs-antibiotics

The development of bacterial resistance to antibiotics is currently one of the primary concerns, which limits the options to treat severe infections. Combination therapy is believed to be an easy approach to boosting the effectiveness of existing antibiotics and thereby tackling antimicrobial resistance. The ability of NDOs to augment the effectiveness of conventional antibiotics against *E. coli, S. aureus,* and *S. agalactia* was examined in this thesis (**Chapter 3** and **4**). AOS was chosen in combination with antibiotics against *S. agalactia,* because AOS have the ability to inhibit *S. agalactia* growth. We showed that AOS (2 and 4%) sensitizes *S. agalactia* to trimethoprim (TMP) by significantly lowering the effective TMP concentration, resulting in an effect similar to that obtained with a more than eight times higher TMP concentration (**Chapter 4**). Despite the fact that only TMP caused significant *S. agalactia* sensitization, 4% AOS diminished the lowest effective concentration of clindamycin (CLI) by 4-fold (from 0.125 to 0.0313g/ml CLI).

Furthermore, both AOS and COS were chosen in combination with antibiotics against *S. aureus* because they exhibited the capacity to fully inhibit *S. aureus* biofilm formation. COS demonstrated synergistic and additive activities against *S. aureus* biofilm formation when

combined with CLI (the ribosomal protein 50S subunits targeting agent) and tetracycline (TET) (the ribosomal protein 30S subunits targeting agent), respectively. An additive interaction was observed when AOS was combined with both antibiotics. Interestingly, when AOS and COS were combined with two bactericidal antibiotics (ampicillin (AMP) and ciprofloxacin (CIP)), the effects were indifferent or even antagonistic. The ability of different NDOs (AOS, COS, FOS, GOS) to augment the action of ampicillin against *E. coli* was investigated in another Chapter (**Chapter 3**) of this thesis. Interestingly, the combination of low concentrations of AOS (0.5 and 1 µg/ml) plus ampicillin resulted in a 2-fold reduction in the minimum inhibitory concentration (MIC) of AMP against *E. coli*. In addition, findings from an anti-adhesion assay revealed that combining AOS (with anti-adhesive properties against *E. coli*) with ampicillin boosted the efficacy of an antibiotic. On the other hand, the combination of other NDOs with ampicillin had no particular additional impact against *E. coli* (**Chapter 3**).

There are some indications that can explain the mechanisms behind the effectiveness of the combinations mentioned above. COS may reduce biofilm formation via ionic interactions (48) and CLI is likely to limit exoprotein formation in *S. aureus* biofilms because COS inhibit bacterial protein synthesis by binding to the 50S component of the bacterial rRNA inside the cell. Hu *et al*. (2019) investigated the effects of sub-inhibitory CLI on *S. aureus* exoprotein expression and found that sub-inhibitory CLI concentrations significantly reduce the *S. aureus* biofilm exoprotein (49). In addition, the additive effect found when AOS and COS interact with TET might be due to a similar mode of action, as TET limits protein synthesis by binding to the 30S subunit of *S. aureus*. Findings on the capacity of AOS to augment the efficiency of antibiotics against *S. agalactia* revealed an increasing trend. This trend, however, is mostly due to the efficacy of AOS, rather than the extra impact of combined compounds. Furthermore, the results of sensitization of *S. agalactia* to TMP in combination with AOS showed that probably AOS exerts antimicrobial activity via a bacteriostatic pathway. Paolo *et al.* (2014) proved that the reduction of bacterial growth produced by a bacteriostatic compound should result in an overall reduction of efficacy when the compound is used in combination with a bactericidal agent (50).These new insights into the effects of AOS may open up new opportunities for developing treatments for *S. agalactia*-related infections, which currently have few effective treatments. In addition, results from an anti-adhesion study demonstrated that the combination of AOS and AMP was effective against *E. coli* (**Chapter 3**). This might be the result of the bactericidal action of AMP in combination with the anti-adhesion potential of AOS. Furthermore, negatively charged AOS have been shown to effectively scavenge positive ions such as Ca^{+2} and Fe^{+2}. These ions are involved in bacterial cell structure preservation, transport, motility and adhesion (51). It is possible that AOS, via chelating Ca^{+2} and Fe^{+2}, impact bacterial stability, subsequently increasing the antimicrobial activity of AMP.

Future perspectives

Anti-adhesion properties against pathogens, inhibition of biofilm formation, anti-inflammatory properties during infection, inhibition of pathogen growth, toxin-binding properties and prevention of intestinal barrier disruption, are among the anti-pathogenic functionalities revealed by NDOs in this thesis. Several mechanisms of anti-pathogenic activity have been described, however, further investigation is still required. We need to determine how NDOs can help to prevent evasion (biofilm), invasion (growth and adhesion), intestinal barrier disruption and inflammation caused by bacterial pathogens (**Chapter 3**, **4** and **6**) in more detail. Investigation of the role of monomers/building blocks of NDOs will help in achieving a better understanding of structure-function relationships of NDOs. For example, investigation of the COS monomers: Glucosamine or N-Acetylglucosamine, may increase our understanding of the modes of action of COS. Furthermore, determining the electrokinetic potential of the pathogen surface after COS supplementation may help in confirming our hypothesis on how COS exert anti-biofilm properties against *S. aureus*. Examining the effect of pH changes on NDO activity and efficiency might help us figure out whether the NDO-related effects are due to NDO-induced pH changes. Besides, changes in pH from 6 to 7.5 can also be used to examine whether the NDOs-related effects are consistent across the small and large intestines. It is also crucial to understand why the antibacterial activity of NDOs (specifically AOS) against pathogenic bacteria is strain- and concentration-dependent (**Chapter 3**, **4** and **7**). Understanding why only certain AOS concentrations inhibit *E. coli* growth and lead to decreased binding of Shiga toxin to its receptors would also shed light on the mechanisms of AOS in anti-bacterial infection (**Chapter 3** and **7**). Investigation of the interaction between Toll-like receptors (and possible other NDO receptors) and NDOs (AOS, COS, GOS) can help us to improve our knowledge about the anti-inflammatory properties of NDOs (**Chapter 3**). As in **Chapter 7** of the thesis, interesting data from an ELISA experiment pointed out that NDOs (COS and AOS) and glycopolymer 9 can partially and fully, respectively, inhibit the binding of Shiga toxin1 B to the Gb3 receptor, therefore examining the effects of NDOs and Glycopolymer 9 on shiga toxin (AB5) translocation across intestinal epithelial cells (**Chapter 7**) can confirm our ELISA results. Furthermore, this thesis warrants further investigation of the mechanisms of action related to the observed effects of combination NDO-antibiotic treatments (**Chapter 3** and **4**). Eventually, *in vivo* models can be used to confirm the above-mentioned capabilities of NDOs in real-life infections.

The diversity of antimicrobial properties and minimal reported side effects make NDOs an attractive tool in the fight against emerging infections and antibiotic resistance. Based on these potentials, which are usually found in the gut, it is strongly recommended to assess the capacity of NDOs in other organs, such as the skin and lungs. Systemic stability, toxicity, and immunogenicity of NDOs must be tested before NDOs can be used as therapeutics against bacterial infections alone or in combination with antibiotics. Furthermore, the impact of NDOs on boosting beneficial bacteria in the gut should not be overlooked as a well-balanced microbiota contributes to infection prevention by suppressing pathogenic bacteria and/or coordinating appropriate immune responses.

References

1. Nina Parker, Mark Schneegurt, Anh-Hue Thi Tu, Philip Lister BMF. Microbiology [Internet]. OpenStax. OpenStax; 2016. Available from: https://openstax.org/books/microbiology/pages/1-introduction
2. Schwertmann, A. , Schroten, H. , Hacker, J. & Kunz C. S-Fimbriae From Escherichia coli Bind to Soluble Glycoproteins From Human Milk. J Pediatr Gastroenterol Nutr. 1999;28(3):257–63.
3. Roilides E, Simitsopoulou M, Katragkou A, Walsh TJ. How Biofilms Evade Host Defenses. Microbiol Spectr. 2015;3(3).
4. Arciola CR, Campoccia D, Montanaro L. Implant infections: Adhesion, biofilm formation and immune evasion. Nat Rev Microbiol [Internet]. 2018;16(7):397–409. Available from: http://dx.doi.org/10.1038/s41579-018-0019-y
5. Asadpoor M, Ithakisiou G.N PAJH, Pieters R, Folkerts G, Braber and S. Non-Digestible Oligosaccharides and Short Chain Fatty Acids as Therapeutic Targets against Enterotoxin-Producing Bacteria and Their Toxins. Toxins (Basel). 2021;13(3):175.
6. Brodsky IE, Medzhitov R. Targeting of immune signalling networks by bacterial pathogens. Nat Cell Biol. 2009;11(5):521–6.
7. Love MJ, Bhandari D, Dobson RCJ, Billington C. Potential for bacteriophage endolysins to supplement or replace antibiotics in food production and clinical care. Antibiotics. 2018;7(1):1–25.
8. Swennen K, Courtin CM, Delcour JA. Non-digestible oligosaccharides with prebiotic properties. Crit Rev Food Sci Nutr. 2006;46(6):459–71.
9. Camilleri M. Human intestinal barrier: Effects of stressors, diet, prebiotics, and probiotics. Clin Transl Gastroenterol. 2021;12(1):1–8.
10. Guarino MPL, Altomare A, Emerenziani S, Di Rosa C, Ribolsi M, Balestrieri P, et al. Mechanisms of action of prebiotics and their effects on gastro-intestinal disorders in adults. Nutrients. 2020;12(4):1–24.
11. Asadpoor M, Peeters C, Henricks PAJ, Varasteh S, Pieters RJ, Folkerts G, et al. Anti-pathogenic functions of non-digestible oligosaccharides in vitro. Nutrients. 2020 Jun;12(6):1789.
12. Rose EC, Odle J, Blikslager AT, Ziegler AL. Probiotics, prebiotics and epithelial tight junctions: A promising approach to modulate intestinal barrier function. Int J Mol Sci. 2021;22(13).
13. Hu X, Jiang X, Gong J, Hwang H, Liu Y, Guan H. Antibacterial activity of lyase-depolymerized products of alginate. J Appl Phycol. 2005 Jan;17(1):57–60.
14. Craft KM, Thomas HC, Townsend SD. Sialylated variants of lacto-: N -tetraose exhibit antimicrobial activity against Group B *Streptococcus*. Org Biomol Chem. 2019;17(7):1893–900.
15. Craft KM, Townsend SD. 1-Amino-2'-fucosyllactose inhibits biofilm formation by *Streptococcus* agalactiae. J Antibiot (Tokyo). 2019 Jun;72(6):507–12.
16. Yildirim-Aksoy M, Beck BH, Zhang D. Examining the interplay between *Streptococcus* agalactiae, the biopolymer chitin and its derivative. Microbiologyopen. 2019 May;8(5).
17. Jeon Y, Park P, Kim S. Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr Polym. 2001;44(1):71–6.
18. Zheng LY, Zhu JF. Study on antimicrobial activity of chitosan with different molecular weights. Carbohydr Polym. 2003 Dec;54(4):527–30.
19. Moon J-S, Kim H-K, Koo HC, Joo Y-S, Nam H, Park YH, et al. The antibacterial and immunostimulative effect of chitosan-oligosaccharides against infection by Staphylococcus aureus isolated from bovine mastitis. Appl Microbiol Biotechnol. 2007 Jul;75(5):989–98.
20. Lin S-B, Chen S-H, Peng K-C. Preparation of antibacterial chito-oligosaccharide by altering the degree of deacetylation of β-chitosan in a *Trichoderma harzianum* chitinase-hydrolysing process. J Sci Food Agric. 2009 Jan;89(2):238–44.
21. Benhabiles MS, Salah R, Lounici H, Drouiche N, Goosen MFA, Mameri N. Food Hydrocolloids Antibacterial activity of chitin , chitosan and its oligomers prepared from shrimp shell waste. Food Hydrocoll [Internet]. 2012;29(1):48–56. Available from: http://dx.doi.org/10.1016/j.foodhyd.2012.02.013
22. Jeon YJ, Kim SK. Production of chitooligosaccharides using an ultrafiltration membrane reactor and their antibacterial activity. Carbohydr Polym. 2000 Feb;41(2):133–41.

23. Shoaf K, Mulvey GL, Armstrong GD, Hutkins RW. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic escherichia coli to tissue culture cells. Infect Immun. 2006;74(12):6920–8.
24. Piotrowski M, Wultańska D, Obuch-Woszczatyński P, Pituch H. Fructooligosaccharides and mannose affect Clostridium difficile adhesion and biofilm formation in a concentration-dependent manner. Eur J Clin Microbiol Infect Dis. 2019 Oct;38(10):1975–84.
25. Petri Jr WA, Smith RD, Schlesinger PH, Murphy CF, Ravdin JI. Isolation of the galactose-binding lectin that mediates the in vitro adherence of Entamoeba histolytica. J Clin Invest. 1987 Nov;80(5):1238–44.
26. Flemming H-C, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2010 Sep;8(9):623–33.
27. Asadpoor M, Ithakisiou GN, van Putten JPM, Pieters RJ, Folkerts G, Braber S. Antimicrobial Activities of Alginate and Chitosan Oligosaccharides Against Staphylococcus aureus and Group B *Streptococcus*. Front Microbiol. 2021;12(September).
28. Dominguez DC. Calcium signalling in bacteria. 2004;54:291–7.
29. Li Y, Liu H, Xu Q, Du Y, Xu J. Chitosan oligosaccharides block LPS-induced O-GlcNAcylation of NF-κB and endothelial inflammatory response. Carbohydr Polym. 2014;99:568–78.
30. Kivit S De, Kraneveld AD, Garssen J, Willemsen LEM. Glycan recognition at the interface of the intestinal immune system : Target for immune modulation via dietary components. Eur J Pharmacol [Internet]. 2011;668:S124–32. Available from: http://dx.doi.org/10.1016/j.ejphar.2011.05.086
31. Ortega-gonz M, Ocon B, Romero-calvo I, Anzola A, Guadix E, Zarzuelo A, et al. Nondigestible oligosaccharides exert nonprebiotic effects on intestinal epithelial cells enhancing the immune response via activation of TLR4-NFκB. Mol Nutr Food Res. 2014;58(2):384–93.
32. Camilleri M, Enteric C, Translational N, Clinic M. The Leaky Gut: Mechanisms, Measurement and Clinical Implications in Humans. 2020;68(8):1516–26.
33. Akbari P, Braber S, Alizadeh A, Verheijden KAT, Schoterman MHC, Kraneveld AD, et al. Galacto-oligosaccharides protect the intestinal barrier by maintaining the tight junction network and modulating the inflammatory responses after a challenge with the mycotoxin deoxynivalenol in human caco-2 cell. J Nutr. 2015;145(7):1604–13.
34. Kivit S De, Kraneveld D, Knippels LMJ. Intestinal epithelium-derived galectin-9 is involved in the immunomodulating effects of nondigestible oligosaccharides. J Innate Immun. 2013;5:625–38.
35. Zenhom M, Hyder A, Vrese M De, Heller KJ, Roeder T. Prebiotic oligosaccharides reduce proinflammatory cytokines in intestinalcCaco-2 cells via activation of PPARγ and peptidoglycan recognition protein 3. J Nutr. 2011;141(5):971–7.
36. Cai Y, Folkerts J, Folkerts G, Maurer M, Braber S. Microbiota-dependent and -independent effects of dietary fibre on human health. Br J Pharmacol. 2020;177(6):1363–81.
37. Ringel Y. Using Probiotics in Gastrointestinal Disorders. Am J Gastroenterol Suppl. 2012;1.
38. Matsuzaki T, Chin J. Modulating immune responses with probiotic bacteria. Immunol Cell Biol. 2000 Feb;78(1):67–73.
39. Wan J, Zhang J, Yin H, Chen D, Yu B, He J. Ameliorative effects of alginate oligosaccharide on tumour necrosis factor-α-induced intestinal epithelial cell injury. Int Immunopharmacol [Internet]. 2020;89(August):107084. Available from: https://doi.org/10.1016/j.intimp.2020.107084
40. Wan J, Zhang J, Xu Q, Yin H, Chen D, Yu B, et al. Alginate oligosaccharide protects against enterotoxigenic Escherichia coli-induced porcine intestinal barrier injury. Carbohydr Polym. 2021;270(March).
41. Yousef M, Pichyangkura R, Soodvilai S, Chatsudthipong V, Muanprasat C. Chitosan oligosaccharide as potential therapy of inflammatory bowel disease: Therapeutic efficacy and possible mechanisms of action. Pharmacol Res [Internet]. 2012;66(1):66–79. Available from: http://dx.doi.org/10.1016/j.phrs.2012.03.013
42. Olivier S, Leclerc J, Grenier A, Viollet MFB, Tamburini J. Ampk activation promotes tight junction assembly in intestinal epithelial caco-2 cells. Int J Mol Sci. 2019;20(20).
43. Dingle T, Wee S, Mulvey GI, Greco A, Kitova EN, Sun J, et al. Functional properties of the carboxy-terminal host cell-binding domains of the two toxins, TcdA and TcdB, expressed by Clostridium difficile. Glycobiology. 2008;18(9):698–706.

44. Bergan J, Dyve Lingelem AB, Simm R, Skotland T, Sandvig K. Shiga toxins. Toxicon. 2012;60(6):1085–107.
45. Su C, Brandt LJ. Escherichia coli 0157 : H7 Infection in Humans. Ann Intern Med. 1998;(36).
46. El-Hawiet A, Kitova EN, Klassen JS. Recognition of human milk oligosaccharides by bacterial exotoxins. Glycobiology. 2015;25(8):845–54.
47. Olano-Martin E, Williams MR, Gibson GR, Rastall RA. Pectins and pectic-oligosaccharides inhibit Escherichia coli O157:H7 Shiga toxin as directed towards the human colonic cell line HT29. FEMS Microbiol Lett. 2003 Jan;218(1):101–5.
48. Khan F, Pham DTN, Oloketuyi SF, Manivasagan P, Oh J, Kim YM. Chitosan and their derivatives: Antibiofilm drugs against pathogenic bacteria. Vol. 185, Colloids and Surfaces B: Biointerfaces. Elsevier B.V.; 2020. p. 110627.
49. Hu, Ramezanpour, Hayes, Liu, Psaltis, Wormald, et al. Sub-Inhibitory Clindamycin and Azithromycin reduce S. aureus Exoprotein Induced Toxicity, Inflammation, Barrier Disruption and Invasion. J Clin Med. 2019 Oct;8(10):1617.
50. Ocampo PS, Lázár V, Papp B, Arnoldini M, Zur Wiesch PA, Busa-Fekete R, et al. Antagonism between bacteriostatic and bactericidal antibiotics is prevalent. Antimicrob Agents Chemother. 2014;58(8):4573–82.
51. Jeon YJ, Park PJ, Kim SK. Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr Polym. 2001 Jan;44(1):71–6.

Appendices

Thesis summary

Despite effective prevention and control efforts over the past few decades, infectious diseases continue to pose a serious threat to global public health, causing millions of deaths annually. Numerous antibiotics can treat bacterial infections. However, practically almost all antimicrobial drugs now have some level of resistance, creating a severe global health issue and the urgent and rapid development of antibiotic alternatives. Non-digestible oligosaccharides (NDOs) may serve as an alternative. NDOs are considered as prebiotics since they are known to specifically promote the growth and/or activity of beneficial bacteria in the intestine, particularly Bifidobacteria and Lactobacilli. However, there is considerable evidence that health-promoting effects of NDOs go beyond helping to modify the intestinal microbiota and immune responses related to the microbiota.

It has been demonstrated that NDOs are significant inhibitors of the development of pathogenic infections. Several anti-pathogenic effects can be achieved by NDOs, depending on their type and structural characteristics. The most frequently mentioned effect is the capacity to serve as a decoy receptor, preventing pathogen adherence. Other pathogenic inhibitory strategies, such as tampering the integrity of the biofilm and the pathogenic cell membrane as well as DNA transcription, have been less studied but may have comparable effects. A full review of the various anti-pathogenic activities of NDOs and related pathways is discussed in **chapter 2**. All anti-pathogenic actions are categorized into a framework, and the structural requirements for an oligosaccharide to produce one of these effects are revealed.

In **chapter 3**, we studied the direct effects of NDOs (alginate-oligosaccharides (AOS), chitosan-oligosaccharides (COS), galacto-oligosaccharides (GOS), and fructo-oligosaccharides (FOS)) on *E. coli. E.coli* is a bacterium that is commonly found in the gut of humans, most *E. coli* strains are harmless, but some strains can cause serious food poisoning which is generally self-limiting, but may lead to severe symptoms and even life-threatening complications, such as hemolytic uremic syndrome. Here, we examined the effect of NDOs on *E. coli* proliferation, adhesion, and inflammatory responses of intestinal epithelial HT-29 cells using the minimum inhibitory concentration (MIC) assay, anti-adhesion assay, and ELISA, respectively. These NDOs were also tested *in vitro* in combination with ampicillin for their effects on *E. coli* growth, adhesion, and the inflammatory response (IL-8 release) of HT-29 intestinal epithelial cells. AOS inhibited the growth of *E. coli* at concentrations of 0.5% and 1%, while high GOS concentrations (6%, 8%, and 10%) had the same impact. It is interesting to note that the combination of low concentrations of AOS and ampicillin (2 g/mL) caused a 2-fold reduction in the MIC level of ampicillin against *E. coli*. The adhesion of *E. coli* to HT-29 cells was also decreased by AOS in a concentration-dependent pattern. These anti-adhesive effects were further enhanced when ampicillin and AOS were combined. The *E. coli*-induced release of IL-8 was markedly inhibited when HT-29 cells were pre-incubated with AOS, COS, or GOS.

In **chapter 4**, we investigated the possibility of using AOS and COS as an alternative to, or in combination with, antibiotic treatment for *Staphylococcus aureus* (*S. aureus*) and *Streptococcus agalactiae* (GBS), which can result in life-threatening infections in both humans and animals at a global scale. These pathogens can cause a wide spectrum of invasive diseases ranging from neonatal sepsis, meningitis, and pneumonia to severe mastitis in cattle. In this study we demonstrated that AOS (2–16%) significantly reduced GBS growth through determining the MIC. Both AOS (8 and 16%) and COS (2 - 16%) were effective in preventing *S. aureus* from forming biofilms. Co-administration of COS and clindamycin showed a synergistic inhibitory effect on the production of *S. aureus* biofilms in a checkerboard biofilm assay. Furthermore, through a series of MIC assays we demonstrated that AOS (2 and 4%) was capable of sensitizing GBS to trimethoprim. In conclusion, AOS and COS affect the proliferation of GBS and *S. aureus* and can function as anti-biofilm agents. The positive effects of AOS and COS in combination with different antibiotics may present novel chances to tackle antimicrobial resistance.

In **chapter 5,** we focused on enterotoxin-producing bacteria (EPB). We compiled a detailed overview of the strategies used by EPB and corresponding enterotoxins to decrease host cell immunity. We also explored the effects of NDOs and short chain fatty acids (SCFA) on EPB functions and offered insight into the possible use of these compounds as effective anti-enterotoxin agents. EPB have developed multiple mechanisms to disrupt gut homeostasis and initiate different pathologies. The release of virulence factors, such as enterotoxins, is assumed to play a significant role in bacterial cytotoxicity. Enterotoxins can invade the intestinal epithelium, depending on their structure and manner of action, leading to long-term effects such hemorrhagic colitis. Numerous NDOs and SCFA interact with enteropathogens and their toxins, which may result in the inhibition of the bacterial pathogenicity. NDOs characterized by diverse structural features, either directly prevent EPB pathogenicity by restricting bacterial adhesion, growth, or biofilm formation, or indirectly by fostering the gut microbiota. Apart from these abilities, NDOs and SCFA can also interact with enterotoxins to lessen their cytotoxicity. These activities primarily depend on their ability to mimic the structure of toxin receptors, hence preventing toxin adhesion to host cells.

In **chapter 6,** the antipathogenic and intestinal barrier-protective abilities of AOS and COS against *Clostridioides difficile* and its major toxin, *clostridium difficile* toxin A (TcdA), were studied. The most prevalent pathogen that causes nosocomial diarrhea in developing countries is *C. difficile*. Besides, *C. difficile* infection (CDI) is expected to become the most common healthcare-associated infection worldwide. Although COS, but not AOS, significantly decreased bacterial growth, the MIC level was not reached. Both oligosaccharides significantly reduced TcdA-induced Caco-2 cell death, according to cell cytotoxicity tests. As determined by the TEER and LY flux assays, exposing Caco-2 monolayers with increasing TcdA concentrations enhanced paracellular permeability in a concentration- and time-dependent trend, but did not cause the release of IL-8. In

this experimental setup, the negative effects of TcdA on the integrity of the monolayer were entirely eliminated and reduced concentration-dependently by COS and AOS, respectively. None of the TcdA- or NDO-stimulated groups showed changes in the expression of tight junction proteins, Zona occludens 1 (ZO-1) and occludin. However, AOS and COS both accelerate the re-assembly of tight junctions according to a calcium-switch assay. These oligosaccharides could perhaps contribute to improved barrier integrity when exposed to TcdA.

In **chapter 7,** we investigated some strategies for preventing the shiga toxin from binding to receptors. Shiga toxin is an AB5 toxin produced by the Shigella species as well as by shiga toxin-producing *E. coli* (STEC). Infection caused by shiga toxin can lead to bloody diarrhea followed by the often fatal hemolytic uremic syndrome. In this study, we sought to develop a simple and efficient toxin inhibitor. Due to this goal, we evaluated the shiga toxin binding inhibitory potentials of three groups of carbohydrate-based inhibitors—glycodendrimers, glycopolymers, and oligosaccharides. We observed a clear enhancement in potency for the multivalent inhibitors, with the divalent and tetravalent compounds inhibiting in the millimolar and micromolar range, respectively. However, the polymeric inhibitor based on galabiose was the most potent in the series exhibiting nanomolar inhibition. COS promisingly and AOS slightly decreased the binding of shiga toxin to the receptor and may be useful in controlling Shigella and STEC epidemics in the future.

In conclusion, we investigated the effects of NDOs (AOS, COS, FOS and GOS) on the defense against several common bacterial pathogens (*E. coli, S. agalactia, S. aureus,* and *C. difficile*) in different steps of the pathogenesis: bacterial growth, bacterial adhesion, biofilm formation, inflammation and bacterial toxin activity. Each of these NDOs had some unique potentials against particular pathogens. Likewise, COS can be described as a potent biofilm inhibitor against *S. aureus*, a strong inhibitor of shiga toxin binding to its receptor (Gb3), a great inhibitor of cytotoxicity and cytopathogenicity of TcdA, an effective anti-inflammatory agent against inflammation caused by *E. coli* with intestinal barrier-protective properties and an enhancer of human intestinal epithelial cell integrity. Besides, AOS can be defined as a powerful inhibitor of the growth of both GBS and *E. coli*, an effective inhibitor of the adhesion of *E. coli* to intestinal epithelial cells, a potent anti-inflammatory agent against inflammation induced by *E. coli*, and an effective mediator on TcdA's ability to disrupt the intestinal barrier. The knowledge about the antimicrobial strategies of NDOs was used to further investigate in combination therapy by combining NDO with antibiotics to increase bacterial sensitivity to specific antibiotics. Likewise, a combination of COS and clindamycin was successful in preventing the growth of a *S. aureus* biofilm, as was a combination of AOS and ampicillin in the case of *E. coli* growth. AOS and trimethoprim were also successful in treating GBS when combined.

NDOs are a desirable weapon in the fight against pathogens and antibiotic resistance due to their variety of antibacterial capabilities and minimal reported adverse effects.

Based on NDOs antimicrobial potentials, which are mostly observed in the gut, it is highly advised to evaluate the capability of these compounds in other organs, such as the skin and lungs. Furthermore, it is necessary to investigate NDOs systemic stability, toxicity, and immunogenicity before they can be employed as treatments against bacterial infections either alone or in combination with antibiotics. Moreover, a healthy microbiota plays a vital role in infection prevention by inhibiting pathogenic bacteria and/or coordinating proper immune responses, therefore the effect of NDOs on promoting beneficial bacteria in the gut should not be ignored.

Nederlandse Samenvatting

Ondanks inspanningen voor effectieve preventie en bestrijding van infectieziekten de afgelopen decennia, blijven infectieziekten een ernstige bedreiging vormen voor de wereldwijde volksgezondheid met jaarlijks miljoenen doden tot gevolg. Er zijn verschillende antibiotica die bacteriële infecties kunnen behandelen. Helaas hebben bijna alle antimicrobiële geneesmiddelen nu een zekere mate van resistentie, waardoor een ernstig wereldwijd gezondheidsprobleem ontstaat en de urgente en snelle ontwikkeling van alternatieven voor antibiotica noodzakelijk is. Niet-verteerbare oligosachariden zouden een goed alternatief kunnen zijn voor antibiotica. Deze oligosachariden worden beschouwd als prebiotica, omdat bekend is dat ze specifiek de groei en/of activiteit van nuttige bacteriën in de darm bevorderen, met name Bifidobacteriën en Lactobacilli. Er is echter aanzienlijk bewijs dat de gezondheidsbevorderende effecten van oligosachariden verder gaan dan het beïnvloeden van de microbiota in de darm en immuunreacties gerelateerd aan de microbiota.

Het is aangetoond dat oligosachariden belangrijke remmers zijn van de ontwikkeling van pathogene infecties. De anti-pathogene effecten van oligosachariden zijn afhankelijk van hun type en structurele kenmerken. Oligosachariden kunnen bijvoorbeeld als "lokreceptor" dienen, waardoor de aanhechting van ziekteverwekkers wordt voorkomen. Andere mogelijke strategieën van oligosachariden om pathogenen te remmen zoals het beïnvloeden van 1) de integriteit van de biofilm, 2) de celmembraan van het pathogeen, 3) DNA-transcriptie, zijn minder bestudeerd, maar kunnen vergelijkbare effecten hebben. Een volledig overzicht van de verschillende anti-pathogene activiteiten van oligosachariden en mogelijke mechanismes gebaseerd op oligosacharide type en structuur worden besproken in Hoofdstuk 2.

In Hoofdstuk 3 onderzochten we de directe effecten van verschillende niet-verteerbare oligosachariden (alginaat-oligosachariden (AOS), chitosan-oligosachariden (COS), galacto-oligosachariden (GOS) en fructo-oligosachariden (FOS)) op *Escherichia coli (E. coli)*. *E.coli* is een bacterie die vaak wordt aangetroffen in de darmen van mensen, de meeste *E.coli*-stammen zijn onschadelijk, maar sommige stammen kunnen ernstige voedselvergiftiging veroorzaken die over het algemeen vanzelf overgaat, maar kan leiden tot ernstige symptomen en zelfs levensbedreigende complicaties, zoals hemolytisch-uremisch syndroom (HUS). In deze studie hebben we gekeken naar de effecten van oligosachariden op *E.coli* groei, adhesie en ontstekingsreacties (interleukine-8 (IL-8) productie) van darmepitheelcellen (HT-29 cellen) geïnduceerd door *E. coli*, met behulp van de minimale remmende concentratie (MIC) assay, anti-adhesie assay en ELISA. AOS (0.5% en 1%) remde de groei van *E. coli* terwijl hoge GOS concentraties (6%, 8% en 10%) hetzelfde effect hadden. De adhesie van *E. coli* aan HT-29 cellen werd ook verminderd door AOS in een concentratie-afhankelijk patroon. De door *E. coli* geïnduceerde afgifte van IL-8 werd duidelijk geremd wanneer AOS, COS of GOS werden toegevoegd aan de HT-29 cellen. Deze oligosachariden werden ook *in vitro* getest in combinatie met een antibioticum (ampicilline) en effecten op

de *E. coli* groei, adhesie en de ontstekingsreacties van darmepitheelcellen geïnduceerd door E. coli werden opnieuw onderzocht. Het is interessant om op te merken dat de combinatie van lage concentraties AOS en ampicilline (2 g/mL) een tweevoudige verlaging van de minimale remmende concentratie van ampicilline tegen *E. coli* veroorzaakte. Effecten op adhesie werden ook versterkt wanneer ampicilline en AOS werden gecombineerd.

In **Hoofdstuk 4** onderzochten we de mogelijkheid om AOS en COS te gebruiken als alternatief voor, of in combinatie met antibioticabehandeling voor *Staphylococcus aureus* (*S. aureus*) en *Streptococcus agalactiae* (GBS). Deze pathogenen kunnen wereldwijd levensbedreigende infecties veroorzaken bij zowel mensen als dieren. Deze ziekteverwekkers kunnen een breed spectrum van invasieve ziekten veroorzaken, variërend van neonatale sepsis, meningitis en longontsteking tot ernstige mastitis bij runderen.

In deze studie hebben we aangetoond dat AOS (2–16%) de GBS groei aanzienlijk verminderde in de MIC assay. Zowel AOS (8% en 16%) als COS (2 - 16%) waren effectief in het voorkomen van vorming van *S. aureus* biofilms. Zeer interessant was de bevinding dat de gelijktijdige toediening van COS en het antibioticum clindamycine een synergetisch remmend effect hadden op de vorming van *S. aureus* biofilms gemeten in een checkerboard biofilm assay. Bovendien hebben we via een reeks MIC assays aangetoond dat AOS (2 en 4%) in staat was om GBS gevoeliger te maken voor het antibioticum trimethoprim. Concluderend, AOS en COS beïnvloeden de groei van GBS en *S. aureus* en kunnen functioneren als anti-biofilm agentia. De positieve effecten van AOS en COS in combinatie met verschillende antibiotica kunnen nieuwe kansen bieden om antimicrobiële resistentie aan te pakken.

In **Hoofdstuk 5** hebben we ons gericht op enterotoxine-producerende bacteriën (EPB). We hebben een gedetailleerd overzicht samengesteld van de strategieën die door EPB en overeenkomstige enterotoxinen worden gebruikt om de immuniteit van gastheercellen te verminderen. Tevens onderzochten we de effecten van niet-verteerbare oligosachariden en korteketenvetzuren (SCFA) op verschillende functies van EPB en gaven inzicht in het mogelijke gebruik van deze structuren als effectieve middelen tegen enterotoxinen. EPB heeft meerdere mechanismen ontwikkeld om de darmhomeostase te verstoren en ziektes te initiëren. Aangenomen wordt dat het vrijkomen van virulentiefactoren, zoals enterotoxinen, een belangrijke rol speelt bij bacteriële cytotoxiciteit. Enterotoxinen kunnen het darmepitheel binnendringen, afhankelijk van hun structuur en manier van werken, wat leidt tot langetermijneffecten zoals hemorragische colitis. Verschillende niet-verteerbare oligosachariden en SCFA kunnen interactie aangaan met enteropathogenen en hun toxines, wat kan resulteren in de remming van de bacteriële pathogeniteit. Niet-verteerbare oligosachariden met diverse chemische structuren voorkomen ofwel direct EPB-pathogeniteit door bacteriële adhesie, groei of biofilmvorming te beperken, of indirect door de darmmicrobiota te bevorderen. Oligosachariden en SCFA kunnen ook interactie aangaan met enterotoxinen om hun cytotoxiciteit te verminderen. Deze activiteiten

hangen in de eerste plaats af van hun vermogen om de structuur van toxinereceptoren na te bootsen, waardoor de hechting van toxine aan gastheercellen wordt voorkomen.

In Hoofdstuk 6 werden de anti-pathogene en darmbarrière beschermende eigenschappen van AOS en COS tegen *Clostridioides difficile* (*C. difficile*) en zijn belangrijkste toxine Clostridium difficile toxine A (TcdA), bestudeerd. In ontwikkelingslanden is *C. difficile* is de meest voorkomende ziekteverwekker die in het ziekenhuis opgelopen (nosocomiale) diarree veroorzaakt. Bovendien wordt verwacht dat *C. difficile* infecties wereldwijd de meest voorkomende zorggerelateerde infectie zal worden. In deze studie, verminderde COS, maar niet AOS, de *C. difficile* groei aanzienlijk. Beide oligosachariden verminderden de door TcdA geïnduceerde celdood (darmepitheelcellen). Het blootstellen van darmepitheelcellen (Caco-2 cellen) aan toenemende TcdA concentraties verhoogde de doorlaatbaarheid van de darmepitheellaag in een concentratie- en tijdsafhankelijke trend, maar veroorzaakte niet de afgifte van het pro-inflammatoire ontstekingseiwit IL-8. De negatieve effecten van TcdA op de integriteit van de darmepitheellaag werden volledig geëlimineerd en concentratieafhankelijk verminderd door respectievelijk COS en AOS. TcdA of incubatie met oligosachariden vertoonde geen veranderingen in de expressie van tight junction eiwitten (*eiwitcomplexen* die de epitheliale cellen samenhouden), zoals zona occludens-1 (ZO-1) en occludine, maar AOS en COS hadden wel de capaciteit om het herstel van tight junction eiwitten te bespoedigen gemeten met een calcium-switch-assay. Deze oligosachariden zouden wellicht bij kunnen dragen aan het versterken van de darmbarrière bij blootstelling aan TcdA.

In Hoofdstuk 7 hebben we enkele strategieën onderzocht om te voorkomen dat de Shiga-toxine zich aan receptoren bindt. Shiga-toxine is een AB5 toxine geproduceerd door Shigella bacteriën en Shiga-toxine producerende *E. coli* stammen (STEC). Infecties veroorzaakt door Shiga-toxine kunnen leiden tot bloederige diarree, gevolgd door het vaak fatale hemolytisch-uremisch syndroom (HUS). In deze studie hebben we geprobeerd een eenvoudige en efficiënte Shiga-toxineremmer te ontwikkelen en zijn de bindingsremmende mogelijkheden van drie groepen op koolhydraten gebaseerde remmers geëvalueerd: glycodendrimeren, glycopolymeren en oligosachariden. Er was een duidelijke versterking van de potentie van de multivalente remmers, waarbij de tweewaardige en vierwaardige verbindingen remmen in respectievelijk het millimolaire en micromolaire bereik. De polymere remmer op basis van galabiose was echter de meest krachtige in de reeks die nanomolaire remming vertoonde. COS remde veelbelovend, en AOS verminderde, de binding van Shiga-toxine aan de receptor en kan in de toekomst nuttig zijn bij het beheersen van Shigella- en STEC-epidemieën.

Conclusie

In dit proefschrift hebben we de effecten onderzocht van verschillende niet-verteerbare oligosachariden (AOS, COS, FOS en GOS) op de afweer tegen verschillende veelvoorkomende bacteriële pathogenen (*E. coli*, *S. agalactia*, *S. aureus* en *C. difficile*) in verschillende stappen

van de pathogenese: bacteriegroei, bacteriële adhesie, vorming van biofilm, ontsteking en activiteit van bacteriële toxinen. Er werd aangetoond dat deze oligosachariden elk op hun eigen manier een anti-pathogene capaciteit lieten zien. COS kan worden beschreven als een krachtige remmer van *S. aureus* biofilmvorming, een sterke remmer van de binding van Shiga-toxine aan zijn receptor (Gb3), een remmer van cytotoxiciteit van TcdA, een effectief middel tegen ontsteking veroorzaakt door *E. coli* met darmbarrièrebeschermende eigenschappen. AOS kan worden gedefinieerd als een krachtige remmer van de groei van zowel GBS als *E. coli*, een effectieve remmer van de hechting van *E. coli* aan darmepitheelcellen, een krachtig middel tegen ontsteking veroorzaakt door *E. coli*, en een effectief middel voor bescherming van een TcdA-verstoorde darmbarrière. De kennis over de deze antimicrobiële strategieën van niet-verteerbare oligosachariden werd gebruikt om verder onderzoek te doen naar combinatietherapie van oligosachariden met antibiotica om de bacteriële gevoeligheid voor specifieke antibiotica te verhogen. De combinatie van COS en clindamycine was bijvoorbeeld veelbelovend bij het voorkomen van de groei van een *S. aureus* biofilm, evenals de succesvolle combinatie van AOS en ampicilline in het remmen van *E. coli* groei. AOS en trimethoprim waren in combinatie ook succesvol bij de behandeling van GBS.

Niet-verteerbare oligosachariden zijn een veelbelovend wapen in de strijd tegen ziekteverwekkers en antibioticaresistentie vanwege hun verscheidenheid aan antibacteriële eigenschappen en minimale hoeveelheid gerapporteerde bijwerkingen. Op basis van de antimicrobiële effecten van oligosachariden, die meestal in de darm worden waargenomen, wordt het ten zeerste aanbevolen om het vermogen van deze verbindingen in andere organen, zoals de huid en de longen, te evalueren. Bovendien is het noodzakelijk om de systemische stabiliteit, toxiciteit en immunogeniciteit van oligosachariden te onderzoeken voordat ze kunnen worden gebruikt als behandelingen tegen bacteriële infecties, alleen of in combinatie met antibiotica. Bovendien speelt een gezonde microbiota een vitale rol bij infectiepreventie door pathogene bacteriën te remmen en/of goede immuunreacties te coördineren, daarom mag het effect van niet-verteerbare oligosachariden op het bevorderen van nuttige bacteriën in de darm niet worden genegeerd.

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List of publications:

1- **Mostafa Asadpoor**, Casper Peeters, Paul A. J. Henricks, Soheil Varasteh, Roland J. Pieters, Gert Folkerts and Saskia Braber. Anti-pathogenic functions of non-digestible oligosaccharides *in vitro.* Nutrients. 2020 Jun 16; https://doi.org/10.3390/nu12061789.

2- **Mostafa Asadpoor**, Soheil Varasteh, Roland Pieters, Gert Folkerts, Saskia Braber. Differential effects of oligosaccharides on the effectiveness of ampicillin against *Escherichia coli in vitro.* PharmaNutrition. 2021 Jun 1; https://doi.org/10.1016/j.phanu.2021.100264.

3- **Mostafa Asadpoor**, Georgia-Nefeli Ithakisiou, Jos P. M. van Putten, Roland J. Pieters, Gert Folkerts, Saskia Braber. Antimicrobial activities of alginate and chitosan oligosaccharides against *Staphylococcus aureus* and Group B *Streptococcus*. Frontiers in Microbiology. 2021 Sep 13; https://doi: 10.3389/fmicb.2021.700605.

4- **Mostafa Asadpoor**, Georgia-Nefeli Ithakisiou, Paul A. J. Henricks, Roland Pieters, Gert Folkerts and Saskia Braber. Non-digestible oligosaccharides and short chain fatty acids as therapeutic targets against Enterotoxin-producing bacteria and their toxins. Toxins. 2021 Feb 25; https://doi.org/10.3390/toxins13030175.

5- **Mostafa Asadpoor**, Maria Eleni Mavrogeni, Karin Strijbis, Roland J. Pieters, Gert Folkerts, Saskia Braber. Protective effects of alginate and chitosan oligosaccharides against *Clostridioides difficile* bacteria and toxin. Ready to submit

6- Diksha Haksar, **Mostafa Asadpoor**, Torben Heise, Jie Shi, Saskia Braber, Gert Folkerts, Lluis Ballell, Janneth Rodrigues, Roland J. Pieters. Fighting Shigella by blocking its disease-causing toxin. Journal of Medical Chemistry. 2021 Apr 28; https://doi.org/10.1021/acs.jmedchem.1c00152.

7- Maria Eleni Mavrogeni**, Mostafa Asadpoor**, Paul AJ Henricks, Ali Keshavarzian, Gert Folkerts, Saskia Braber. Direct action of non-digestible oligosaccharides against a leaky gut. Nutrients. 2022 Nov 07; https://doi.org/10.3390/nu14214699.

8- **Mostafa Asadpoor**, Masoud Ansarin, Mahboob Nemati. Amino acid profile as a feasible tool for determination of the authenticity of fruit juices. Advanced Pharmaceutical Bulletin. 2014 Aug 10; https://doi: 10.5681/apb.2014.052.

9- Alireza Garjani, Hassan Rezazadeh, Sina Andalib, Mojtaba Ziaee, Yousef Doustar, Hamid Soraya, Mehraveh Garjani, Arash Khorrami, **Mostafa Asadpoor**, and Nasrin Maleki-Dizaji. Ambivalent Effects of Atorvastatin on Angiogenesis, Epidermal Cell Proliferation and Tumorgenesis in Animal Models. Iranian Biomedical Journal. 2012 Apr 14; https://doi: 10.6091/IBJ.1017.2012.