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

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ABSTRACT

Background: 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.

Methods: 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 were determined *in vitro* in the presence or absence of ampicillin, using minimum inhibitory concentration (MIC) assay, anti-adhesion assay and ELISA, respectively.

Results: 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.

Conclusion: Current study highlights the direct effects of NDOs on*E. coli* growth, adhesion and inflammatory responses of HT-29 cells *in vitro*.

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

Abbreviations: AOS, alginate-oligosaccharides; COS, chitosan-oligosaccharides; GOS, galacto-oligosaccharides; FOS, fructo-oligosaccharides; 2′-FL, 2′-Fucosyllactose; MOS, Mannan-oligosaccharides; MIC, minimum inhibitory concentration; NDOs, Non-digestible oligosaccharides; *E. coli*, *Escherichia coli*; TSB, tryptic soy broth; OD, optical density; FCS, fetal calf serum; EDTA, ethylene diamine tetra-acetic acid; DMEM, Dulbecco's modified Eagle's minimum essential medium; ARC, adhesive rate constant; PPARγ, peroxisome proliferator-activated receptor γ; PGlyRP3, peptidoglycan recognition protein 3.

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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 min to reach an optical density (OD) of 0.5 based on McFarland standard (equal to 4×10^8 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 1.5×10^5/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.

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%–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 = 4 × 108 CFU/mL) measured at 600 nm. To polypropylene round-bottom 96-well plates 25 μL from the serial dilutions of oligosaccharides with/without 25 μL ampicillin and 50 μL 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* (2×10^8 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 min 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).

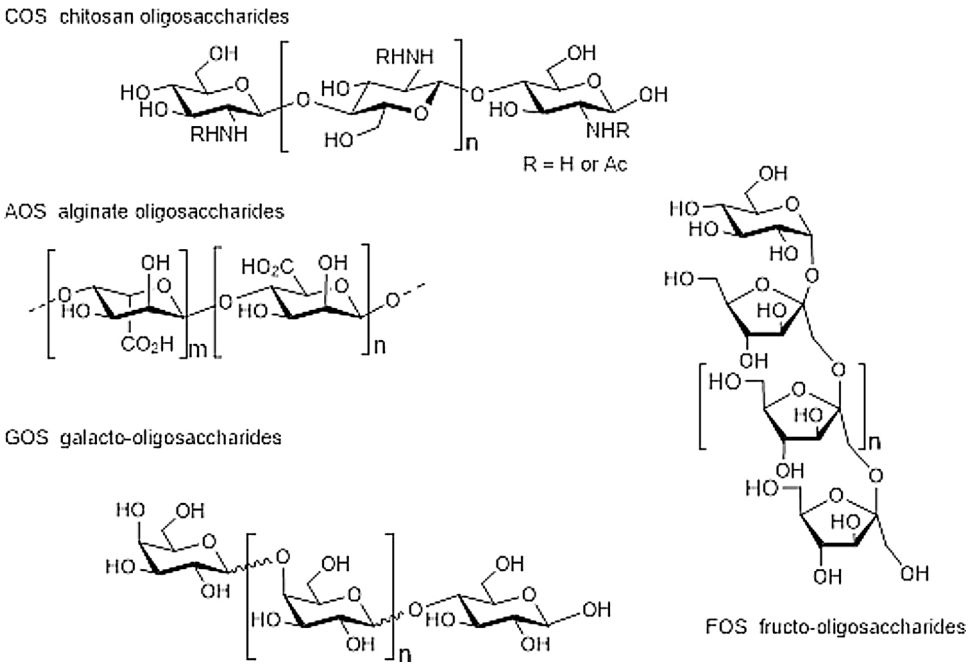


Fig. 1. An overview of the chemical structures of the different NDOs (AOS, COS, FOS, GOS).

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 (2×10^8 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%), 2×10^8 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 $P < 0.05$.

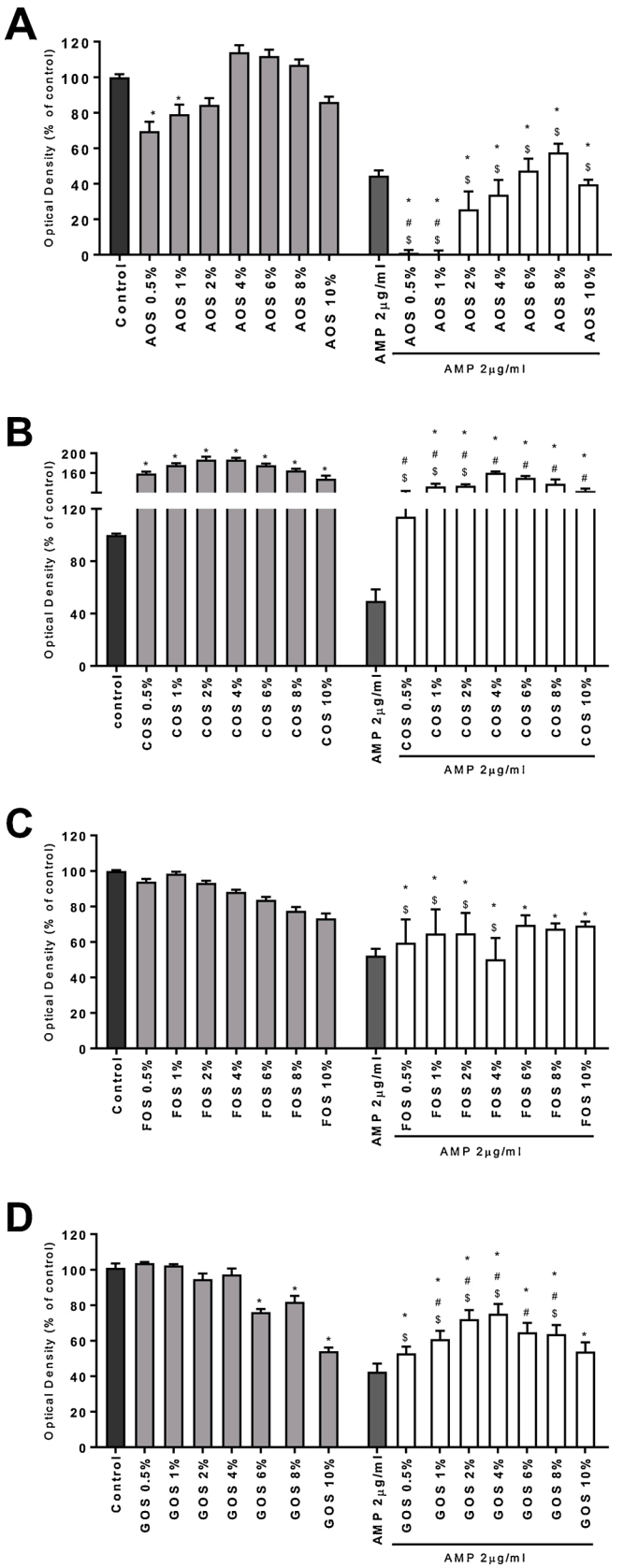
3. Results

3.1. 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 2×10^8 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* (2×10^8 CFU/mL) ± ampicillin (0.5 μg/ mL) for 4 h did not impair HT-29 cell viability (Fig. S3 A–H).

3.2. 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.



(caption on next column)

Fig. 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.

3.3. 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).

3.4. 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. 4 A, 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). Pretreatment 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.

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

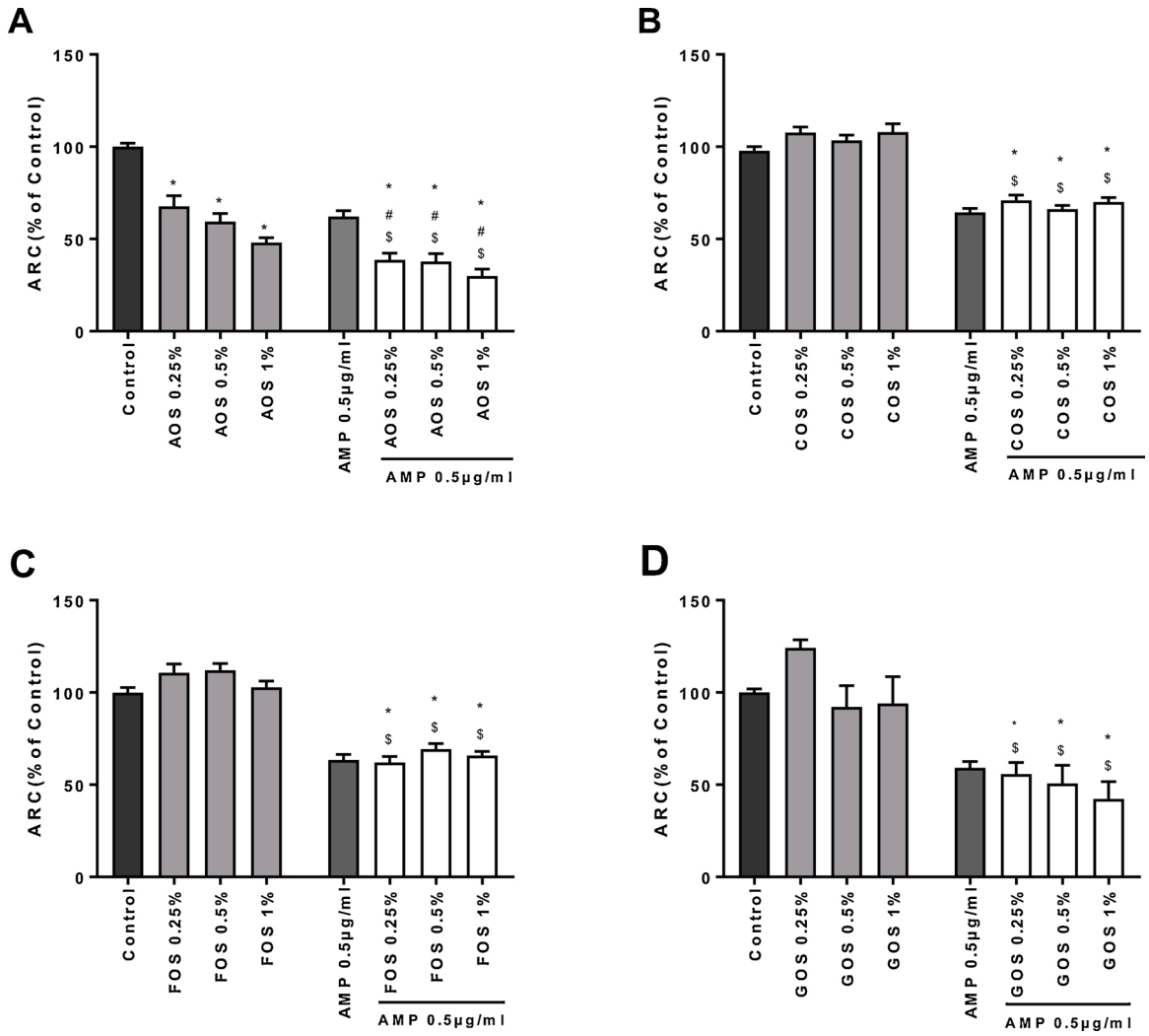


Fig. 3. NDOs with and without ampicillin differentially influence the adhesion of *E. coli* to HT-29 cells.
HT-29 cells, pre-treated in presence or absence of AOS (a), COS (b), FOS (c) and GOS (d) for 24 h, with or without ampicillin and exposed to *E. coli* for 2 h, were lysed and seeded on blood agar 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.

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], 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

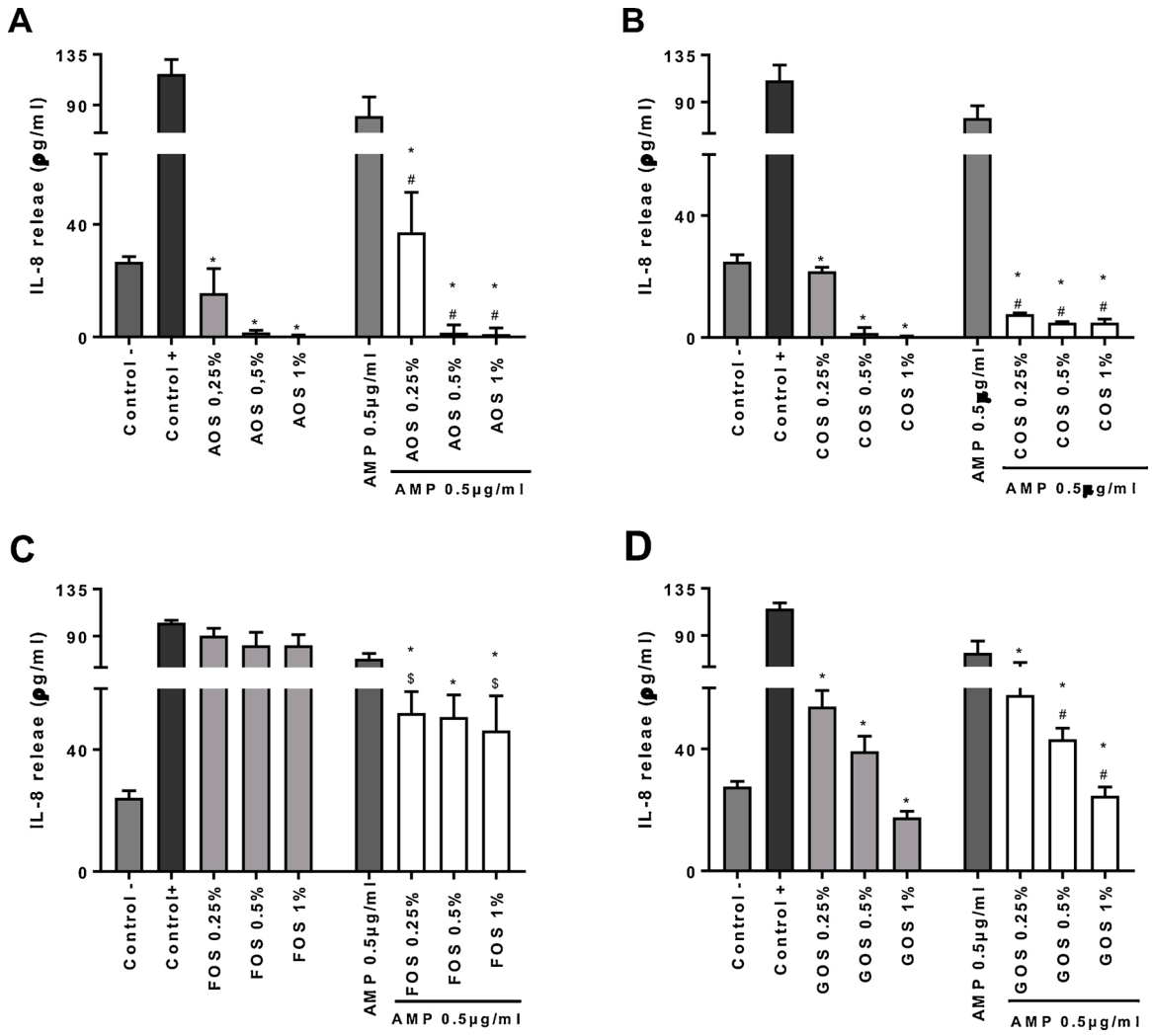


Fig. 4. NDOs from various sources reduce the *E. coli*-induced IL-8 release by HT-29 cells.
HT-29 cells, pre-treated with AOS (a), COS (b), FOS (c) and GOS (d), were exposed to *E. coli* in presence or absence of ampicillin (0.5 ug/mL) and IL-8 release was measured *via* ELISA. Results are expressed 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.

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*.

Author contributions

For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization SB, GF, SV; methodology, SV,SB; software, MA, SV; validation, MA,SV and SB; formal analysis, MA.; investigation, MA,SV; resources, RP,GF; data curation, MA, SV; writing—original draft preparation, MA; writing—review and editing, SA,SB,GF,RP; visualization, MA; supervision, SV,SB; project administration, GF,RP; funding acquisition, GF, RP. All authors have read and agreed to the published version of the manuscript.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phanu.2021.100264.

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