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Basic nutritional investigation

Galactooligosaccharides and 2′-fucosyllactose can directly suppress growth of specific pathogenic microbes and affect phagocytosis of neutrophils

Esmaeil Mortaz Ph.D. [a,*], Masoumeh Nomani Ph.D. [a], Ian Adcock Ph.D. [b,c], Gert Folkerts Ph.D. [d], Johan Garssen Ph.D. [d,e]

[a] Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Priority Research Centre for Asthma and Respiratory Disease, Hunter Medical Research Institute, University of Newcastle, Newcastle, NSW, Australia
[c] Airways Section, National Heart and Lung Institute, Imperial College London, London, United Kingdom
[d] Division of Pharmacology, Faculty of Science, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, Netherlands
[e] Danone Nutricia Research, Utrecht, The Netherlands

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ABSTRACT

Objectives: Non-digestible oligosaccharides such as milk oligosaccharides (MOS) can regulate and influence immune function. As an example, galactooligosaccharides (GOS), and 2′-fucosyllactose (2′-FL; a specific human MOS) regulate immune development and functionality. *Staphylococcus aureus* (SA) and *Pseudomonas aeruginosa* (PA), both serious pathogens, can cause severe and life-threatening infections. The aim of this study was to examine the effects of GOS and 2′-FL on bacterial growth and on polymorphonuclear (PMN) phagocytosis.
Methods: PMNs were isolated from heparinized whole human blood before treatment/incubation with GOS (0.0625−10%), 2′-FL (0.5−2.5%) and/or GOS combined with 2′-FL (GOS 10%/2′-FL 2.5%; GOS 0.0625%/2′-FL 0.5%) and incubation with green florescent protein (GFP)-labeled SA or PA for 60 h. GFP-relative fluorescent units (GFP-RFU) was measured ≤60 h using a plate reader. Bacterial lag time was determined by the time to onset of exponential bacterial fluorescence/growth alone or after co-culture of bacteria and PMN. Viable bacterial colony-forming units (CFUs) were determined after 60 h.
Results: SA and PA growth lag time was suppressed by co-incubation with GOS in a concentration-dependent manner. This was significant for both SA and PA at concentrations >2.5% GOS ($P \leq 0.05$ for both SA and PA) but only for SA at 1% GOS ($P \leq 0.05$). 1.5% 2′-FL significantly suppressed the lag time of SA growth ($P \leq 0.05$) and was effective against SA and PA at 2.5% ($P \leq 0.01$ and $P \leq 0.01$, respectively). GOS (10%, 5%) and 2.5% 2′-FL significantly decreased SA and PA bacterial growth/CFUs ($P \leq 0.05$).
Conclusion: The data suggests that both GOS and 2′-FL can suppress growth of serious pathogens and enhance phagocytosis.

Introduction

Probiotics and prebiotics have gained considerable attention due to their immunomodulatory function both in vitro and in vivo. Prebiotics cause specific changes in the gastrointestinal (GI) microbiome resulting in beneficial effects on host health [1]. Therefore, prebiotics have been proposed as preventative agents for non−antibiotic-associated GI diseases [2].

Galactooligosaccharides (GOS), fructooligosaccharides and inulin are the most common prebiotics tested to date. GOS are non-digestible and are derived from lactose that occurs naturally in mammalian milk and consist of chains of galactose monomers. In contrast, inulin and inulin-type fructans are soluble dietary fibers [3]. GOS contains two to eight saccharide units with one of these units being a terminal glucose and the remaining units being galactose or a disaccharide comprised of two units of galactose [4]. GOS are attractive food additives for infant milk formula because of their ability to modulate the intestinal microbiota, improve intestinal development, enhance mineral absorption, and protect the intestinal barrier [5,9]. Additionally, increasingly more data indicate the unique immunomodulatory effects induced by GOS [6−10].

GOS can promote the growth of beneficial bacteria and improves host human clonic [11−13]. GOS are selectively

*Corresponding author: Fax number:+31 30 253 1599.
E-mail address: emortaz@gmail.com (E. Mortaz).

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fermented in the intestine, resulting in the growth and/or activity of bifidobacteria and lactobacilli [14,15]. In practice, there is indirect evidence showing that GOS may influence the immune function in for example, eczema patients [16] and in the prevention of allergic diseases such as asthma [17]. Importantly, GOS have beneficial effects in atopic dermatitis by controlling *Staphylococcus aureus* (SA)-associated exacerbations [18].

The rationale for using GOS and perhaps other non-digestible oligosaccharides is further enhanced by their ability to stimulate the growth of gut health, promoting microbes (i.e., probiotic function) [19]. Thus, prebiotics could prevent the growth of pathogenic microorganisms and promote the growth of health: promoting microbes (i.e., probiotic species [20,21].

Human milk oligosaccharide (HMO) is the third most abundant solid component of milk and has inhibitory effects on the adhesion of microorganisms to the intestinal mucosa. Moreover, HMO inhibits the growth of pathogens through the production of bacteriocins and organic acids and the expression of genes involved in inflammation management [22,23]. Additionally, internalization of *Escherichia coli* by epithelial cells is significantly reduced by pretreatment with HMO [24]. Additionally, GOS inhibits the attachment of enteropathogenic *Escherichia coli* to HEp-2 and Caco-2 epithelial cell lines [25]. Finally, Xylo-oligosaccharides reduce the attachment of listeria monocytogenes to enterocytes, present in the intestinal lumen, by modulating bacterial surface properties [26].

Prebiotic oligosaccharides, particularly GOS, have structural similarity with cell surface glycoproteins and are postulated to inhibit adhesion of toxins and pathogens to cells [27]. Thus, we hypothesized that as an example GOS and 2′-fucosyllactose (2′-FL) might suppress bacterial growth and affect their elimination by neutrophils. Therefore, we aimed to investigate the effectiveness of GOS and 2′-FL at various concentrations on gram-positive and gram-negative bacterial growth and on neutrophil phagocytosis of these bacteria.

Materials and methods

GOS and 2′-FL preparation

Galactooligosaccharides (Vivinal GOS syrup (VGOS; 45% GOS in dry matter) were provided by Friesland Campina Domo (Borculo, The Netherlands). GOS was diluted in RPMI 1640 complete media containing 10% heat-inactivated fetal bovine serum and 100U/mL penicillin-streptomycin antibiotic solution (Gibco, Thermo Fisher, Waltham, MA, USA) to 0.0625%, 0.125%, 0.25%, 0.5%, 1%, 2.5%, 5%, and 10%. 2′-FL was purchased (Biosynth-Carbosynth, Compton, United Kingdom) and diluted in RPMI 1640 complete media to 0.5%, 1%, 1.5%, and 2.5%. Bacteria or neutrophils were subsequently treated with GOS and 2′-FL at various concentrations for 60 h and during time the intensity of green fluorescent protein (GFP)-labeled SA and *Pseudomonas aeruginosa* (PA) was measured.

Preparation of bacterial strains

The methicillin-resistant SA (MRSA) strain MW2 and PA were used and prepared as described previously [28].

Determination of the effect GOS and 2′-FL on bacterial growth

We cultured 2×10^6 colony-forming units (CFUs)/mL GFP-SA or GFP-PA on a 96-well plate in the presence or absence of GOS (0.0625, 0.125, 0.25, 0.5, 1, 2.5, 5, and 10%) or 2′-fucosyllactose (0.5, 1, 1.5, and 2.5%) as described previously [34] for ≤60 h. The highest and lowest concentrations of GOS (10 and 0.0625%) along with 2′-FL (2.5 and 0.5%) were used to evaluate possible higher/more than additive effects. The levels of GFP, which reflect bacterial growth, was measured by a FLUOstar plate reader.

Isolation of human blood polymorphonuclear cells

Polymorphonuclear (PMN) were isolated from heparinized peripheral blood of five healthy individuals as described previously [28]. After isolation, the cells were resuspended in HEPES III buffer (HEPES, Gibco, Waltham, MA, USA) supplemented with (0.5% w/v bovine serum albumin, 1 mM CaCl2, 5 mM glucose) and counted by microscopy.

Determining PMN phagocytosis and CFUs

Isolated PMN were co-cultured with various concentrations of GOS or 2′-FL together with GFP-PA or GFP-SA in 96-well plates (black, clear-bottom; TC Surface, Thermo Fisher). Briefly, 2×10^6 PMN/mL was cultured in human pooled serum (40% vol/vol; Sigma-Aldrich, St. Louis, MO, USA), HEPES III buffer, and 2×10^6 CFU/mL of bacteria. The plates were placed in a FLUOstar Optima (BMG Labtech, Ortenberg, Germany) at 37°C with constant shaking (150g) for 60 h. Every 20 min, the GFP signal of each well was recorded by a fluorescence plate reader (excitation 485 nm/emission 520 nm) as previously described [29,30]. After 60 h, bacterial suspensions were removed from the plates and 30 μL of the suspension diluted in serum (sterile human saline 0.9% [NaCl] solution) and cultured on UTI-Agar plates (HiCrom-HIMEDIA) overnight at 37°C. Bacterial suspensions from cells exposed to high concentrations of GOS and to 2′-FL were evaluated for CFU as previously described [29].

Statistical analysis

All experiments were performed in triplicate. Results are expressed as mean ± SEM. Data were analyzed by Kruskal-Wallis test using GraphPad Prism 8 software version 8.00. Results were considered statistically significant when $^{*}P \leq 0.05$, $^{\dagger}P \leq 0.01$ and $^{\ddagger}P \leq 0.001$, and $^{\S}P \leq 0.0001$. Results are presented using GraphPad Prism software.

Results

GOS, 2′-FL, and bacterial interaction

GFP-SA or GFP-PA were cultured in the presence or absence of GOS or 2-FL. In some experiments, isolated human PMNs were included in the cultures. The lag time before the bacterial population starts to exponentially in a new environment was calculated as described previously [29]. GOS (1. 2.5, 5, and 10%), in the absence of PMNs, significantly reduced the lag time before GFP-SA started to grow exponentially ($P \leq 0.05$, $P \leq 0.05$, $P \leq 0.001$, and $P \leq 0.0001$, respectively; Fig. 1A, B). 2′-FL (1.5 and 2.5%) reduced the lag time of GFP-SA as well ($P \leq 0.01$ and $P \leq 0.05$, respectively; Fig. 1C, D).

Similar results were observed after incubation of GFP-PA with GOS and 2′-FL. GOS (2.5, 5, and 10%), all significantly suppressed GFP-PA growth compared with that seen with bacteria alone ($P \leq 0.05$, $P \leq 0.001$, and $P \leq 0.001$, respectively; Fig, 2A, B). 2′-FL (2.5%) suppressed the growth of GFP-PA as well ($P \leq 0.01$; Fig. 2C, D).

GOS and 2′-FL enhance antibacterial capacity of PMN

Incubation with PMN (alone) decreased the growth of GFP-SA (Fig. 3). Addition of GOS decreased the growth of GFP-SA compared with GFP-SA and PMN alone in a concentration-dependent manner (Fig. 3A).

The concentration-dependent further suppression of GFP-SA growth with GOS reached significance at 2.5% ($P \leq 0.05$), 5% ($P \leq 0.01$), and 10% ($P \leq 0.01$; Fig. 3B). This was reflected in a significant increase in the lag time at these concentrations (Fig. 3B). 2′-FL further suppressed PMN-induced suppression of GFP-SA growth (Fig. 3C) and significantly increased the lag time at 1.5% ($P \leq 0.05$) and 2.5% ($P \leq 0.05$; Fig. 3D)

GFP-PA growth was suppressed by adding PMNs in a co-culture and was further suppressed by both GOS and 2′-FL in a concentration-dependent manner (Fig. 4). Incubation with GOS (1, 2.5, 5, and 10%) and 2′-FL (0.5, 1, 1.5, and 2.5%) further suppressed PMN-suppressed bacterial growth compared with that seen with PMNs alone (Fig. 4A and C). The effect on lag time reached significance for GOS (5%, $P \leq 0.001$ and 10%, $P \leq 0.01$) and 2′-FL (1.5%, $P \leq 0.01$ and 2.5%, $P \leq 0.05$) compared with the effect with PMNs alone (Fig $P \leq 4$B and D).

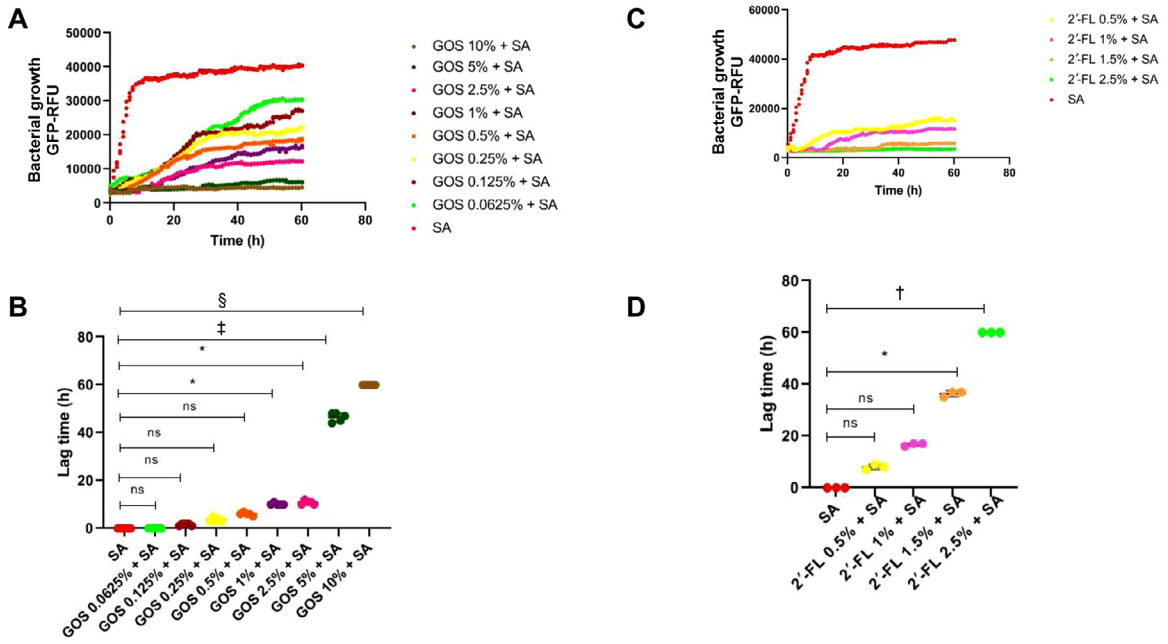

Fig. 1. Effect of GOS and 2′-FL on SA growth. (**A**) Representative growth curves of green fluorescent protein GFP-SA with various concentrations of GOS. (**B**) Graphical analysis of lag time for SA growth in the presence of different concentration of GOS. (**C**) Representative growth curves of green fluorescent protein GFP-SA with various concentrations of 2′-FL. (**D**) Graphical analysis of lag-time for GFP-SA growth in the presence of different concentration of 2′-FL. Results are presented as individual with bars as mean ± SEM. $^{*}P \leq 0.05$, $^{\dagger}P \leq 0.01$ and $^{\ddagger}P \leq 0.001$, and $^{\S}P \leq 0.0001$. 2′-FL, 2′-fucosyllactose; GFP, green fluorescent protein; GOS, galactooligosaccharides; ns, non-significant; SA, *Staphylococcus aureus*.

Determination of the effect of PMN and GOS/2′-FL on bacterial CFU

In the absence of cells, GFP-SA CFU and GFP-PA reached similar levels of growth ($2.7 \pm 0.06 \times 10^9$ versus $2.5 \pm 0.03 \times 10^9$ CFUs; Fig. 5). PMNs alone had no significant effect on either GFP-SA or GFP-PA CFU (Fig. 5). The addition of GOS (5 and 10%, both $P \leq 0.05$; Fig. 5A) and 2′-FL (2.5%, $P \leq 0.05$) significantly reduced the number of GFP-SA CFUs compared with that seen with PMNs alone (Fig. 5B). Similarly, the addition of GOS (5 and 10%, both $P \leq 0.05$; Fig. 5C) or 2′-FL (2.5%, $P \leq 0.05$; Fig. 5D) significantly attenuated the number of GFP-PA CFUs compared with PMNs alone.

Combination effect of GOS and 2′-FL

A combination of the low concentrations of GOS and 2′-FL (0.625 and 0.5%. respectively) did not significantly affect bacterial

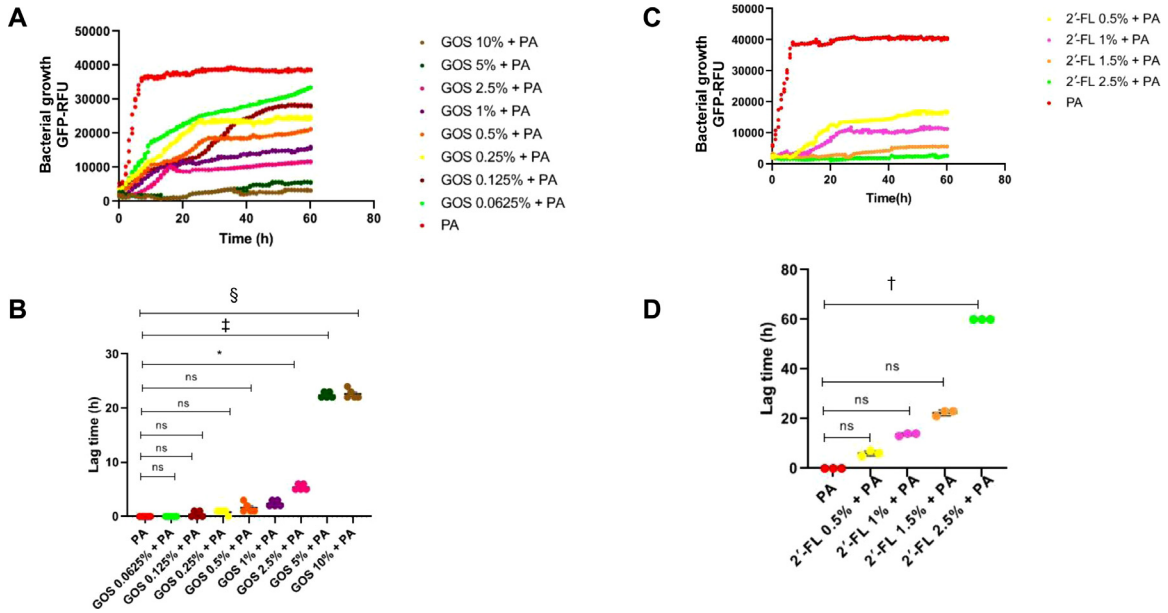

Fig. 2. Effect of GOS and 2′-FL on GFP-PA growth. (**A**) Representative growth curves of GFP-PA and different concentration of GOS. (**B**) Graphical analysis of lag time for GFP-PA growth in the presence of different concentration of GOS. (**C**) Representative growth curves of GFP-PA and different concentration of 2′-FL. (**D**) Graphical analysis of lag time for PA growth in the presence of different concentration of 2′-FL. Results are presented as mean ± SEM. $^{*}P \leq 0.05$, $^{\dagger}P \leq 0.01$, and $^{\ddagger}P \leq 0.001$ show significance between groups. 2′-FL, 2′-fucosyllactose; GFP, green fluorescent protein; GOS, galactooligosaccharides; ns, non-significant; PA, *Pseudomonas aeruginosa*.

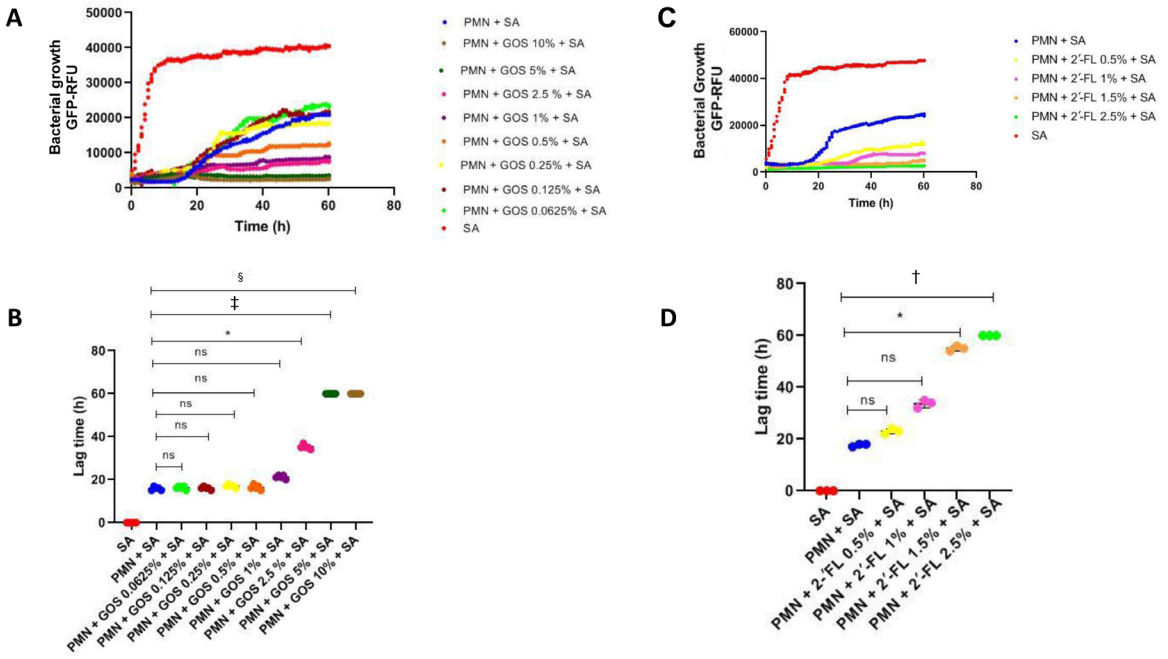

Fig. 3. Effects of GOS and 2′-FL on phagocytosis of bacteria GFP-SA by PMN. SA-GFP incubated with PMN in presence or absence of GOS and 2′-FL at various concentrations for 60 h. Bacterial phagocytosis by PMN was measured by FLUostar. Results are representative of growth curves. (**A**) PMN, GFP-SA, and GOS. (**B**) The individual data at various time points were plotted to the graph and shows the lag time of PMN and GOS and SA-GFP. (**C**) Representative growth curves of GFP- SA and PMN and 2′-FL. (**D**) Graphical analysis of lag time of PMN and 2′-FL and GFP-SA. Results are presented as mean ± SEM. $^{*}P \leq 0.05$, $^{\dagger}P \leq 0.01$ show significance between groups. 2′-FL, 2′-fucosyllactose; GFP, green fluorescent protein; GOS, galactooligosaccharides; ns, non-significant; PMN, polymorphonuclear; SA, *Staphylococcus aureus.*

growth (Fig. 6A) or lag time (Fig. 6B) of GFP-PA. In contrast, the combination of the high concentrations of GOS and 2′-FL (10 and 2.5%, respectively) significantly reduced GFP-SA growth ($P \leq 0.05$) and lag time ($P \leq 0.05$; Fig. 6A, B). These combinations of GOS and 2′-FL had a similar effect on GFP-SA growth (Fig. 6C) and lag time (Fig. 6D) when cultured in the presence of PMNs. There was a

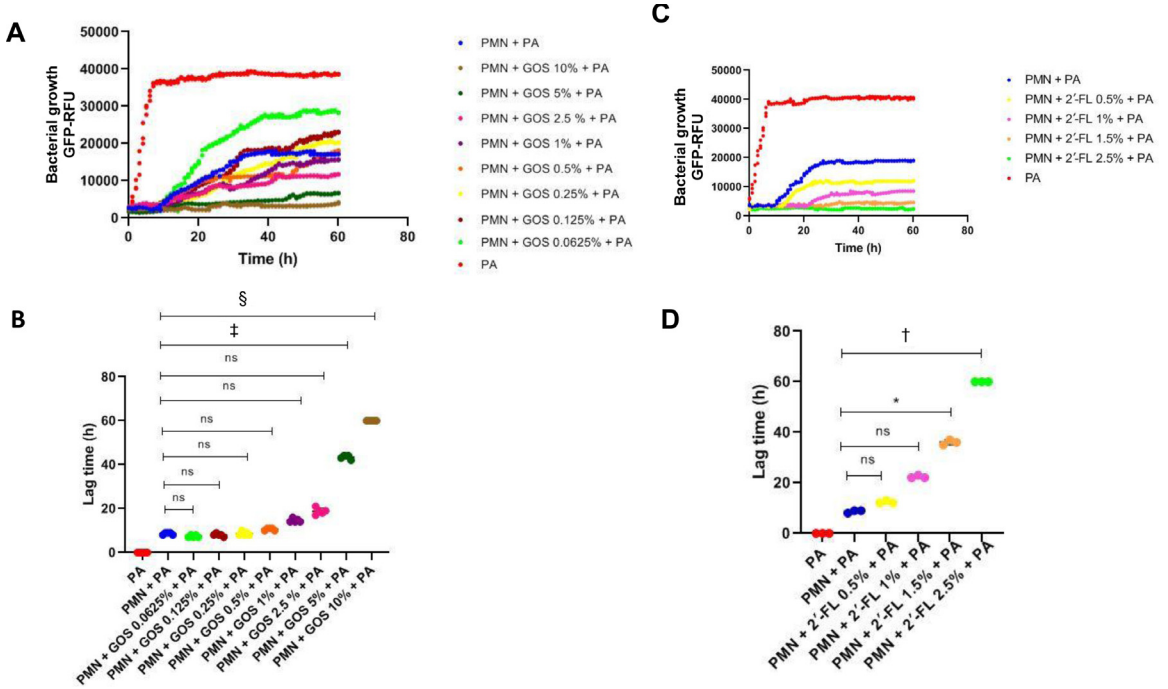

Fig. 4. Effects of GOS and 2′-FL on phagocytosis of bacteria GFP-PA by PMN. PA-GFP bacteria was incubated with fresh isolated PMN in presence or absence of GOS at various concentrations and 2′-FL for 60 h. Bacterial phagocytosis by neutrophils was measured by FLUostar. Results are representative of growth curves (**A**) PMN, PA, and GOS. (**B**) The lag times of growth bacteria GFP-PA, PMN, and GOS were plotted to the graph. (**C**) Graphical analysis of growth of PMN and 2′-FL and PA-GFP. (**D**) Data showing lag time of growth GFP-PA bacteria, PMN, and 2′-FL were plotted to the graph. Results are presented as mean ± SEM. $^{*}P \leq 0.05$, $^{\dagger}P \leq 0.01$, and $^{\ddagger}P \leq 0.001$ show significance between groups. 2′-FL, 2′-fucosyllactose; GFP, green fluorescent protein; GOS, galactooligosaccharides; PA, *Pseudomonas aeruginosa*; PMN, polymorphonuclear.

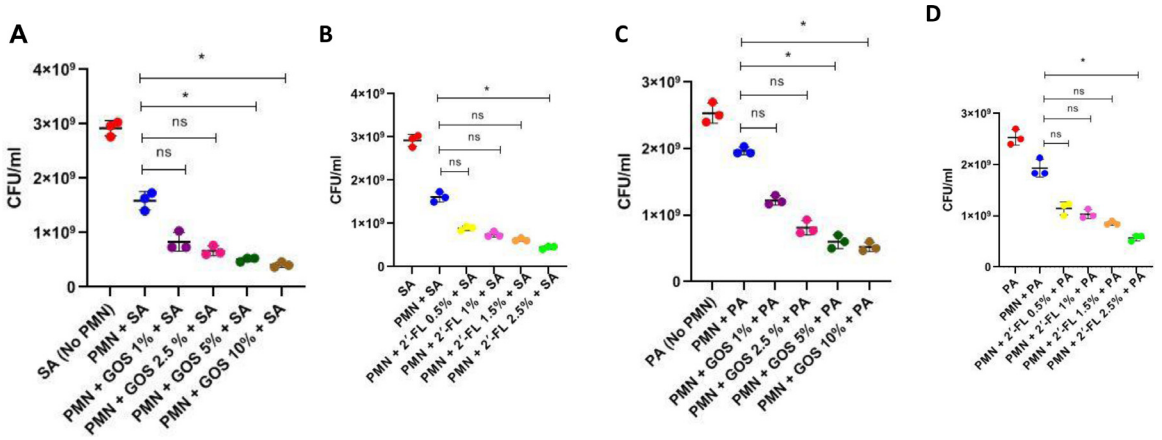

Fig. 5. CFU of bacteria after 60 h incubation with PMN, GOS, and 2′-FL. Bacteria and PMN in presence of GOS/2′-FL was incubated for 60 h and then removed and cultured in UTI medium. (**A**) Growth GFP-SA treated with GOS. (**B**) GFP-SA treated with 2′-FL. (**C**) GFP-PA treated with GOS. (**D**) GFP-PA treated with 2′-FL in the presence or absence of PMN. Results are presented as CFUs/mL. Results are presented as mean ± SEM. *$P \leq 0.05$ shows significance between groups. 2′-FL, 2′-fucosyllactose; CFU, colony-forming unit; GFP, green fluorescent protein; GOS, galactooligosaccharides; PA, *Pseudomonas aeruginosa*; PMN, polymorphonuclear; SA, *Staphylococcus aureus*.

similar effect of high and low combinations of GOS and 2′-FL on bacterial growth (Fig. 6E) and lag time (Fig. 6F) in the absence and presence of PMNs (Fig, 6G and H, respectively).

Discussion

The present study showed that GOS and 2′-FL could suppress the bacterial lag time and growth of SA and PA. Additionally, when combined with neutrophils, bacterial phagocytosis increased. GOS, at both 5% and 10% concentrations, was able to kill both bacteria that were tested.

SA is an opportunistic pathogen and considered an important public health threat. Neutrophils are the major phagocytic cell type in human blood that respond rapidly to infections and have a short half-life of 5.4 d, as demonstrated in in vivo studies [31]. Neutrophils act in two ways to destroy infectious agents or particles namely by releasing and producing reactive oxygen species (ROS) but also by the generation of antimicrobial cytotoxic host defence peptides and proteases within secretory vesicles. Opsonizations of the bacteria by immunoglobulin facilities engulfing the bacteria and requires proteases, amidases, ROS formation for finally destroying the bacteria in a metabolic burst [32].

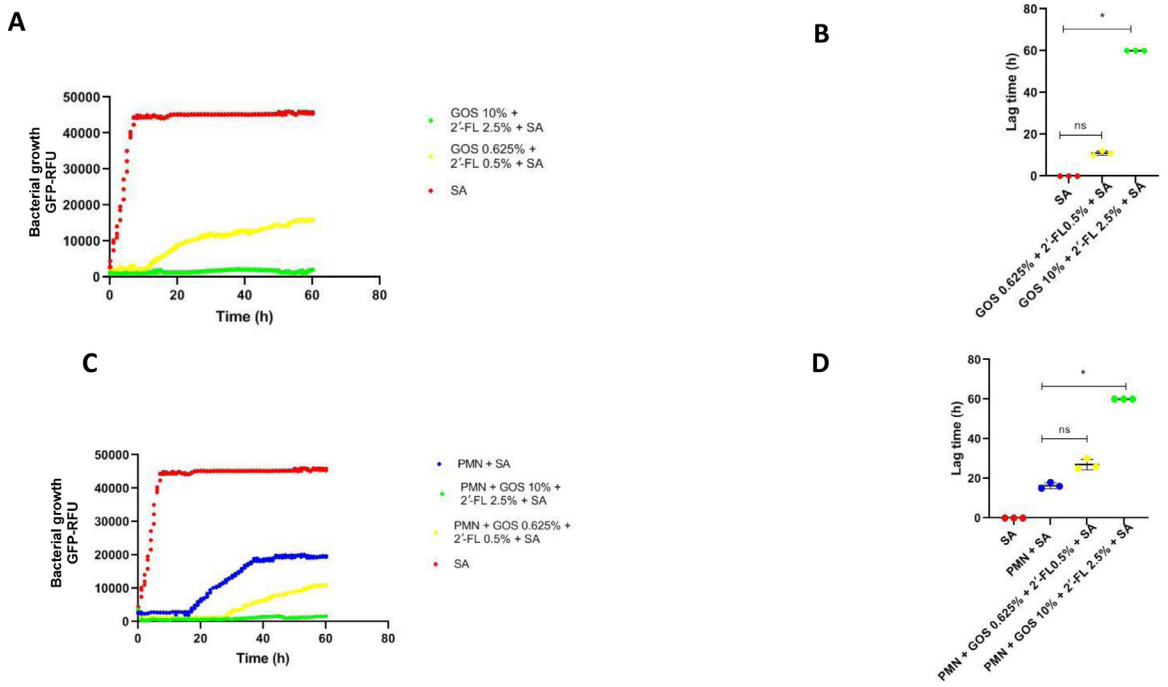

Fig. 6. Effect of the highest and lowest concentrations of GOS and 2′-FL on SA and PA growth. (**A**) Representative growth curves of GFP-SA with GOS and 2′-FL. (**B**) Graphical analysis of lag time for SA growth in the presence of GOS and 2′-FL. (**C**). Representative growth curves of GFP-SA with GOS and 2′-FL. (**D**) Graphical analysis of lag time for GFP-SA growth in the presence of GOS and 2′-FL. Results are presented as individual with bars as mean ± SEM. *$P \leq 0.05$. (**E**) Representative growth curves of GFP-PA with GOS and 2′-FL. (**F**) Graphical analysis of lag- time for PA growth in the presence of GOS and 2′-FL. (**G**) Representative growth curves of GFP-PA with GOS and 2′-FL. (**H**) Graphical analysis of lag time for GFP-PA growth in the presence of GOS and 2′-FL. Results are presented as individual with bars as mean ± SEM. *$P \leq 0.05$. ns is indicating non-significant. 2′-FL, 2′-fucosyllactose; GFP, green fluorescent protein; GOS, galactooligosaccharides; PA, *Pseudomonas aeruginosa*; ns, non-significant; SA, *Staphylococcus aureus*.

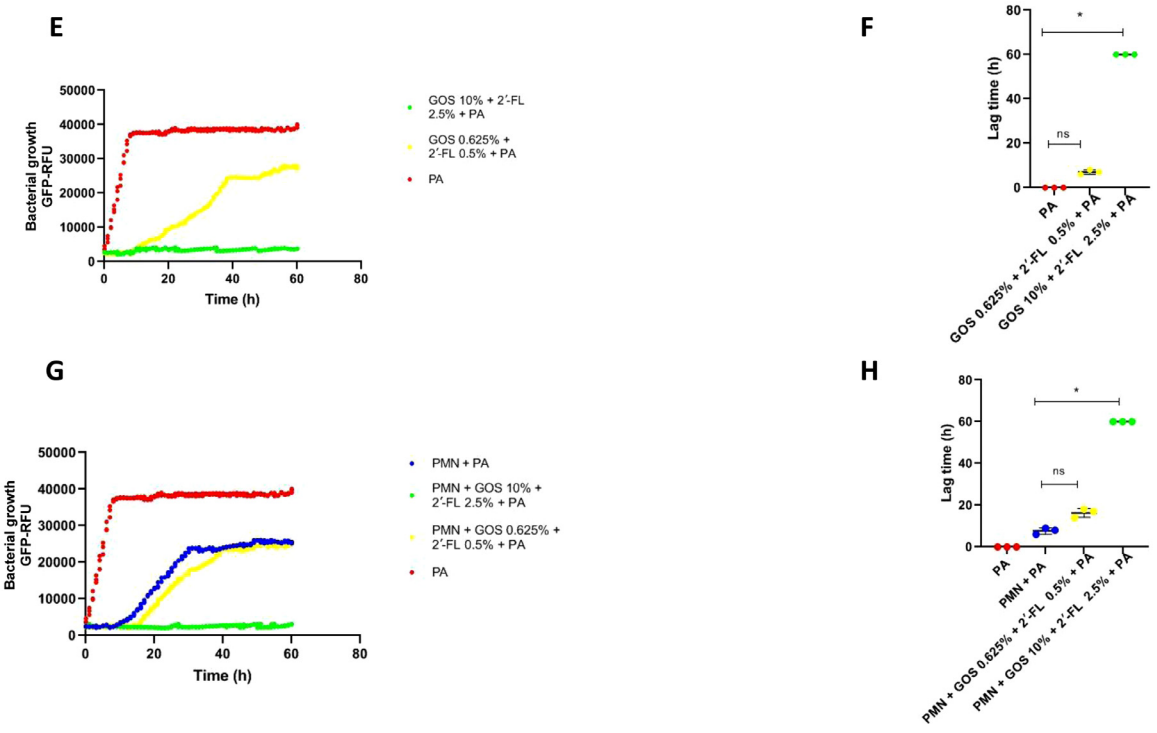

Fig. 6. Continued

Neutrophils have multiple oxygen-independent and -dependent mechanisms to eliminate invading agents. Phagocytosis, for example, is associated with the production of oxygen-free radicals by the enzyme NADPH-oxidase and the integration of cytoplasmic granules within the phagosome containing the pathogen [33]. Neutrophil-mediated killing of bacteria depends on the production of ROS [34]. However, defective ROS generation may allow neutrophils to engulf bacteria but allow bacterial proliferation inside phagosomes [30].

PA is a major cause of nosocomial infections [35]. PA is a common microorganism that causes infections in people with immune deficiencies, burns, and in those with an abnormally low neutrophil levels. Indeed, any innate or acquired immune system dysregulation can result in PA infection [36]. For example, PMNs from patients with cystic fibrosis or chronic granulomatous disease are unable to destroy PA [48]. PMNs remove PA by an intracellular process following phagocytosis as well as by the process of neutrophil extracellular trap formation [37].

The current study shows that both GOS and 2′-FL suppress the growth of both SA and PA but thus far, we did not investigate the mechanism(s) of their action. There is evidence that their antimicrobial effects may be due to dehydration by the oligosaccharides. Bacteria require water for growth and this requirement is termed *water activity* (aw) [38,39]. The addition of sucrose, glucose, or fructose causes an increase in the concentration of the aqueous solution around the microorganism and a change in aw [38,39]. Every microorganism has a limiting aw below which it cannot grow. A low aw produces a high osmotic pressure.

Sugar has been used to treat wounds infected by SA, resulting in inhibition of bacterial growth [38,39]. Studies showed honey, which contains glucose, fructose, sucrose, and trisaccharides, has antibacterial potency [40]. For example, in vivo studies indicate that honey has antibacterial activity against PA and *E. coli* [41] and against biofilms made by MRSA and PA [42]. These effects are thought to occur by enhancing the osmotic pressure resulting in bacterial shrinking [40]. Interestingly, Akiyama et al. showed that GOS can be used topically in cream of skin lesions of atopic dermatitis to prohibit SA exacerbation. Moreover, 5% GOS is able to block glycocalyx production by SA and suppress the colonization of SA [18]. Moreover, another study showed that GOS can moisture retention with a high solubility [43]. Additionally, it has been shown that GOS has high stability and could be used in many commercial goods such as infant formulas, dairy products, breakfast cereals, bakery products, and as a sugar replacement [44,45].

In one study with lactose-intolerant individuals, using GOS increased lactose-fermenting bacteria, which correlated with an improvement of symptoms [46]. Moreover, a study demonstrated that using GOS effectively alleviated constipation and was able to increase lactic acid bacteria and short-chain fatty acid production [47].

Although there are several strengths to the present study, we recognize that there are some limitations, including the limited number of samples and the fact that we used samples from individuals with defects of neutrophil function.

Conclusions

The results from the present study demonstrated that the GOS and 2′-FL have antibacterial properties and can inhibit the growth of pathogenic bacteria and increase the ability of bacteria phagocytosis by neutrophils. These prebiotics may, in the future, be used successfully to modify bacterial infections. However, further studies are required, particularly in relation to the effects of prebiotics on wound healing, where bacterial infection plays a key role.

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