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Bovine milk oligosaccharides as anti-adhesives against the respiratory tract pathogen *Streptococcus pneumoniae*

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ABSTRACT

Streptococcus pneumoniae is a Gram-positive pathogen, which is regularly found in the upper respiratory tract of healthy individuals. Increased numbers of *S. pneumoniae* have been observed colonising the upper respiratory tract of children affected by respiratory tract infections. Galβ1-4GlcNAcβ1-3Gal has been previously identified as one of the receptors involved in the adherence and translocation of *S. pneumoniae*. As this structure is similar to the milk oligosaccharide lacto-N-neoTetraose, many studies have investigated if free milk oligosaccharides can inhibit the adhesion of *S. pneumoniae* to epithelial cells of the respiratory tract. Here, we demonstrate that bovine oligosaccharides, which were extracted from demineralised whey, using a combination of membrane filtration and chromatography, were capable of reducing *S. pneumoniae* adhesion to pharynx and lung cells in vitro when tested at physiological concentrations. This study strengthens the potential use of bovine derived milk oligosaccharides as functional ingredients to reduce the incidence of infectious diseases.

1. Introduction

Respiratory tract infections (RTI) account for almost half of all general practitioner and hospital visits by infants and young children (Bachrach, Schwarz, & Bachrach, 2003). The most common infections include acute otitis media, sinusitis and bronchitis. In general, RTI are caused by either viral or bacterial pathogens and very often as a combination of both. Bacteria frequently associated with respiratory tract infections include *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus* (Bosch, Biesbroek, Trzcinski, Sanders, & Bogaert, 2013; Pettigrew, Gent, Revai, Patel, & Chonmaitree, 2008).

Streptococcus pneumoniae is a Gram positive pathogen, which regularly colonises the upper respiratory tract (URT) of healthy individuals. Carriage of *S. pneumoniae* is typically asymptomatic in nature (Bogaert, de Groot, & Hermans, 2004). However, if this bacterium gains access to the sterile parts of the respiratory tract, the result is a swift inflammatory response, which in turn causes disease. Elevated *S. pneumoniae* colonisation has been recorded in the URT of children suffering from RTI (García-Rodríguez & Martinez, 2002). Furthermore, the colonisation of the respiratory tract by *S. pneumoniae* reduces the microbial diversification of the host; this, in turn, has been linked to an increased risk of respiratory infections. Infections of the lower respiratory tract of infants and young children is also a matter of great importance, as pneumonia is one of the leading causes of global infant mortality. In fact, greater than 30% of pneumonia related deaths are caused by *S. pneumoniae* (Rudan, Boschi-Pinto, Biloglav, Mulholland, & Campbell, 2008).

To cause infection, *S. pneumoniae* must first adhere to human nasopharyngeal epithelial cells. One of the receptors responsible for the attachment of *S. pneumoniae* to human nasopharyngeal epithelial cells is GlcNAcβ1-3Gal (Andersson & Svanborg-Eden,

1989). This receptor shares similarity with the oligosaccharide lacto-N-neoTetraose, Galβ1-4GlcNAcβ1-3Galβ1-4Glc (LNnT) (Idänpään-Heikkilä et al., 1997). In this respect, several studies have demonstrated that synthesised oligosaccharides (OS) inhibit the adhesion of *S. pneumoniae* to epithelial cells of the respiratory tract. For instance, the pre-exposure of *S. pneumoniae* to LNnT and its α2-6-sialylated derivative reduced the pneumococcal load in the lungs of animal models (Idänpään-Heikkilä et al., 1997). Furthermore, LNnT was reported to inhibit the adherence of *S. pneumoniae* to the receptor Galβ1-4GlcNAcβ1-3Gal (Tong, McIver, Fisher, & DeMaria, 1999). LNnT also provided a protective effect against *S. pneumoniae* by preventing pneumonia in rabbits (Idänpään-Heikkilä et al., 1997). Sialylated oligosaccharide ligands terminating in NeuAcα2-3(or 6)Galβ1 were demonstrated to reduce the adhesion of *S. pneumoniae* to human bronchial and tracheal cells (Barthelson, Mobasseri, Zopf, & Simon, 1998). These studies strongly suggest that free OS such as LNnT, 3'SLNnT, 6'SLNnT, can prevent the adhesion of *S. pneumoniae* to human epithelial cells. These OS and several other complex OS are naturally found in breast milk (Kunz, Rudloff, Baier, Klein & Strobel, 2000). In fact, it is known that infants those who are exclusively breastfed have a lower incidence of RTI (Wright, Holberg, Martinez, Morgan, & Taussig, 1989).

However, the protective effects ascribed to human milk oligosaccharides (HMO) are not available to formula-fed infants. Infant milk formulas are based on bovine milk, which contains a lower concentration of bovine milk oligosaccharides (BMO; ~0.03 g L^{-1}) compared to OS in human milk (10–15 g L^{-1}; Kunz et al., 2000). A number of BMO do, however, share the same structure as certain HMO, which could imply common functionalities (Barile et al., 2009; Mariño et al., 2011). Therefore, value may lie in extracting and concentrating BMO with a view to their addition as an active ingredient to infant formulas. In a recent pilot study (Mehra et al., 2014), a powder enriched in BMO was produced through a membrane filtration process using mother liquor as a starting material. In

the current study, this method was used to generate an enriched-BMO powder from demineralised whey powder, which is an important ingredient in infant formula manufacture. The powder was further depleted in lactose through size-exclusion chromatography. The final powder, which was enriched in BMO, was examined for its ability to prevent adhesion of *S. pneumoniae*.to respiratory cells using in-vitro assays.

2. Materials and methods

2.1. Materials

Tissue culture reagents were purchased from Sigma–Aldrich (Wicklow, Ireland) and LGC (Middlesex, United Kingdom). The oligosaccharides 3′-sialyllactose and 6′-sialyllactose (3-SL and 6-SL, respectively) were purchased from Carbosynth, Compton, UK. The purity of both 3-SL and 6-SL is a minimum 98% according to the company's specification.

2.2. Enrichment of oligosaccharides

For enrichment of OS, demineralised whey powder, purchased from Dairygold Co-Operative Society Ltd (Mitchelstown, Ireland), was used in a joint project between Teagasc and University of California, Davis to enrich OS, according to Mehra et al. (2014). Starting with demineralised whey powder, which was re-suspended in water to give a final volume of 2428 L at 5% total solids, the process yielded 2.5 kg of milk oligosaccharide-rich powder (OSP), which was transferred to the Food for Health Ireland consortium and used in the present study, with the agreement of University California, Davis.

For testing OS in biological assays, OSP was further treated to remove residual peptides and large levels of lactose. 50 mL of a 20% solution of the OSP was applied to a

BioGelP2 size exclusion column (Bio-rad Laboratories, Inc., USA; 92 × 5 cm) and eluted with deionised water at a flow rate of 3 mL min^{-1}. The fractions (14 mL) were analysed for lactose, 3-SL and 6-SL using high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and peptide concentration (Bradford, 1976). Peptide-free and low-trace lactose fractions (< 80 mg L^{-1}) from 15 runs were pooled and freeze-dried to give an oligosaccharide-rich fraction (OSF).

2.3. Quantification of lactose and sialyllactose by high performance liquid chromatography

Demineralised whey powder, OSP, OSF and fractions from BioGelP2 were appropriately diluted in water and analysed for quantification of lactose, 3-SL and 6-SL. Lactose in demineralised whey powder, OSP and OSF was quantified by high performance liquid chromatography (HPLC) using an HPX-87C carbohydrate column (300 × 7.8 mm) (Aminex, Bio-Rad, UK) and a refractive index detector. The elution was obtained in isocratic conditions using 4.5 mM sulphuric acid for 30 min. 3-SL and 6-SL in all samples above and lactose in fractions from BioGelP2 were quantified by HPAEC-PAD, according to Mehra et al. (2014).

2.4. Structural characterisation of milk oligosaccharides

The free OS in the OSF were structurally characterised by hydrophobic interaction liquid chromatography (HILIC) coupled to mass spectrometry by the National Institute for Bioprocessing Research & Training (NIBRT, Dublin, Ireland) as described by Mariño et al. (2011).

2.5. Organisms and growth conditions

The *S. pneumoniae* strain ATCC BAA-255 (*S. pneumoniae* R6) was obtained from the American Type Culture Collection. *S. pneumoniae* R6 was stored in Todd Hewitt broth (Becton Dickinson and Company, France) containing 10% (v/v) glycerol at –80 °C and cultured directly from storage into the same broth with 0.5% (w/v) yeast extract (0.2% inoculum) at 37 °C with 5% CO_2 until an optical density (600 nm) of 0.8 was reached.

2.6. Culture of pneumocytes

Adherent Detroit 562 (pharynx) and A549 (lung) cells were purchased from the American Type Culture Collection. These cell lines were chosen because of their routine use in previous studies (Jensch et al., 2010; Kallio et al., 2014). The Detroit 562 cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% (w/v) of penicillin-streptomycin. The A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) of FBS and 1% (w/v) of penicillin-streptomycin. Cells were incubated at 37 °C in an atmosphere of 5% CO_2 and passaged every 3–4 days at ratio 1:6. When the cells were used in the adhesion assay, they were washed twice with PBS and then incubated with 0.25% trypsin/EDTA solution. Trypsination was stopped by adding 8–10 mL of fresh antibiotic-free medium. After seeding cells in 12-well plates to a density of 5×10^5 per well, they were incubated overnight, and then the spent medium was replaced with 1 mL of DMEM or EMEM supplemented with 2% (v/v) FBS. After a further overnight incubation as described above, the cells were used in the adhesion assay.

177

178 *2.7. Adhesion assay*

179

180 The adhesion assay used in the present study was adapted from previous publications
181 (Marotta, Ryan, & Hickey, 2014). Prior to the adhesion assay, confluent monolayers were
182 treated with 5 µg mL^{-1} interleukin 1β for 4 h at 37 °C with 5% CO_2 to mimic host cell
183 response during infection (Rosenow et al., 1997). Briefly, 6 mg mL^{-1} solutions of
184 demineralised whey powder and OSP were prepared in EMEM supplemented with 2% (v/v)
185 FBS. Solutions of OSF were prepared at the following concentrations: 6 mg mL^{-1}, 4 mg mL^{-1},
186 2.4 mg mL^{-1}, 2 mg mL^{-1}, 1.15 mg mL^{-1} and 0.24 mg mL^{-1} (corresponding to 0.3, 0.2, 0.125,
187 0.1, 0.0575 and 0.0125 mg mL^{-1} of 6-SL in OSF, respectively) in the appropriate medium
188 supplemented with 2% FBS (v/v). All solutions were sterilised by filtration. Bacteria were
189 harvested and re-suspended in tissue culture media with or without OS at 1×10^6 colony
190 forming units (cfu) mL^{-1} and incubated for 30 min at 37 °C in an atmosphere of 5% CO_2 (pre-
191 incubation step). Controls with no saccharide were also prepared. Confluent monolayers in
192 12-well plates were washed with PBS, infected with 1 mL of pre-incubated bacteria and
193 incubated for 30 min at 37 °C with 5% CO_2. After 30 min incubation, the wells were washed
194 three times with PBS to remove any non-adherent bacteria and lysed with 1 mL of PBS
195 containing 0.2 % (v/v) Triton X-100 (Sigma, Steinheim, Germany) for 30 min at 37 °C on a
196 shaking platform at 100 agitations per min to ensure maximal recovery of viable bacterial
197 cells. The lysates were serially diluted and enumerated by spread-plating on sheep blood agar
198 plates. Aliquots of the experimental inocula were retained, diluted and plated to determine
199 original cfu mL^{-1}. Agar plates were incubated at 37 °C with 5% CO_2 overnight after which
200 cfu were enumerated.

201

2.7. Bacterial interaction

In an effort to determine if OS interact with bacteria, the adhesion assay was slightly modified. *S. pneumoniae* R6 was grown as described above, re-suspended (1×10^6 cfu mL^{-1}) in an OSF solution at a concentration of 6 mg mL^{-1} and 2.4 mg mL^{-1} when working with Detroit 562 and A549 cells, respectively, in the appropriate medium supplemented with 2% FBS (v/v) and incubated for 30 min at 37 °C 5% CO_2. The samples were centrifuged at 4000 × *g* for 7 min to pellet the bacterial cells. The medium containing unbound oligosaccharides was removed and the bacterial pellet was re-suspended in an equal volume of appropriate medium. Following this the adhesion assay was performed as described above.

2.8. Cell line interaction

To determine if OS mixture interacts with epithelial cells, the adhesion assay was modified. Confluent monolayers of Detroit 562 and A549 cells were washed with PBS and supplemented with 1 mL of OSF solution at a concentration of 6 mg mL^{-1} (Detroit 562) and 2.4 mg mL^{-1} (A549) in the appropriate medium supplemented with 2% FBS (v/v). Controls were performed in the absence of saccharides. The 12 well plates were incubated at 37 °C 5% CO_2 for 30 min. Following incubation, the 12 well plates were washed 5 times with PBS to remove the unbound OS. The confluent monolayers were then infected with 1 mL *S. pneumoniae* R6 (1×10^6 cfu mL^{-1}) resuspended in the appropriate medium supplemented with 2% FBS (v/v) and incubated for 30 min. To determine the amount of adhering bacteria the adhesion assay was performed as described above.

2.9. Statistical analysis

The adhesion assays were carried out on three separate occasions in triplicate. Results are presented as mean ± standard deviations of replicate experiments. Graphs were drawn using Microsoft Excel and the unpaired student t-test was used to determine statistically significant results; $P < 0.05$ was considered significant.

3. Results and discussion

It is widely accepted that human milk protects and promotes infant health (Gartner et al., 2005). For instance, human milk plays a major role in protecting infants from respiratory infections (Duijts, Ramadhani, & Moll, 2009; Wright et al., 1989). Recently, in addition to IgA, free HMO have been implicated in this protective role, which may be exerted through direct and/or indirect effects (Stepans et al., 2006). As direct effect, HMO may interfere with adhesion, by acting as decoys to which pathogens can bind. In the URT, the frequent bathing in milk might modulate the adherence of bacteria to epithelial cells through the high concentration of OS present, thereby reducing the incidence of harmful organisms and lowering the risk of infection (Barthelson et al., 1998). In the lower respiratory tract, OS may reach the respiratory epithelia through absorption into the blood stream, where they could influence bacterial-host interactions in a similar manner as observed in the gut. In this respect, Goehring, Kennedy, Prieto, and Buck (2014) have demonstrated that some ingested HMO are absorbed intact into the infant circulation. In terms of indirect effects, specific HMO may have effect on the immune system, as demonstrated by numerous in vitro studies (Bode et al., 2004a; Bode, Rudloff, Kunz, Strobel, & Klein, 2004b; Eiwegger et al., 2010).

As *S. pneumoniae* is one of the major bacterial etiological agents of respiratory tract infections in infants and children, we focused our attention on investigating the effect of a

pool of oligosaccharides on adhesion of *S. pneumoniae* on respiratory cells of both the upper and lower respiratory tract. As human milk is not available for commercial purposes, bovine milk streams were considered as a suitable source of OS, given their widespread availability.

3.1. Enrichment of oligosaccharides

As previously mentioned, concentrations of OS in bovine milk and its streams are much lower than concentrations of OS found in human milk. For this reason, before testing the biological properties of BMO, they were extracted and concentrated from demineralised whey. Demineralised whey was selected as starting material for OS enrichment, because it contains a higher concentration of sialyllactose (SL, 3'- and 6'- sialyllactose) (47 mg L^{-1}) for similar lactose concentration (48 g L^{-1}) compared with bovine milk. Furthermore, demineralised whey is characterized by lower mineral levels, which may be advantageous for applications in infant formula manufacture, when compared to other bovine streams with similar SL and lactose concentrations (such as whey permeates). To evaluate enrichment of OS through the process, SL was selected as a marker of total OS, since it is the predominant oligosaccharide in the BMO pool and can be quantified by using routine analytical methods.

Following membrane filtration and diafiltration, the diafiltered OS-enriched retentate had a SL to lactose ratio of 1.65%. This represents a 17-fold enrichment of SL based on the SL/Lactose ratio. Upon evaporating and spray drying the retentate, 2.5 kg of a powder (OSP) was obtained with the following composition: 70.21% (w/w) lactose, 1.20% (w/w) SL, 24.5% (w/w) protein and 4.41 % (w/w) ash. Despite the enrichment of OS compared to the initial demineralized whey, the major component of the OSP was still lactose. As it has been previously demonstrated that lactose can interfere with the ability of oligosaccharides to influence bacterial adhesion (Kavanaugh et al., 2013), to further reduce lactose and

277 concentrate OS, the OSP was applied to a size-exclusion chromatography, resulting in 8.83 g
278 of OSF.

279 The chromatographic step removed most of the lactose, while retaining approximately
280 71% of SL (Table 1), resulting in a powder with lactose and SL concentration (ratio 3-SL:6-
281 SL was 3.5:1) of 0.9% and 23% (w/w), respectively. Compared with concentrations found in
282 whey (0.07%, w/w; Marotta et al. unpublished data), this represents an approximate 329 fold
283 SL enrichment in the OSF. Recently, nanofiltration was investigated to enrich BMO from
284 lactose-hydrolysed bovine milk (Altmann et al., 2015) and lactose-hydrolysed colostrum
285 whey permeate (Cohen, Barile, Liu, & de Moura Bell, 2017). Altmann et al. (2015) produced
286 a NF retentate containing 873.23 BMO mg L^{-1}. However, the data reported did not allow
287 calculation of the concentration of BMO as percentage of total solid. Cohen et al. (2017)
288 produced a NF retentate containing 5.96 SL g L^{-1}, which represented 6.7% of total solids.

289 Although the process employed in the present study did not hydrolyse lactose and did
290 not employ NF, as in Altmann et al. (2015) and Cohen et al. (2017), the OSF was
291 characterised by a much higher content of SL (23%, w/w). The oligosaccharide profile of the
292 OSF was analysed by NIBRT. After fluorescently labelling with 2-Aminobenzamide (2AB),
293 the sample was analysed by HILIC. A total of 29 peaks were detected and assigned
294 comparing the Glucose Unit (GU) values obtained with GU values previously published
295 (Mariño et al., 2011). Predominant peaks were 3-SL and 6-SL (taken together 55.2% of total
296 peak area), GalNAc(α1-3)Gal(β1-4)Glc (23.8% of total peak area) followed by Gal(α1-
297 3)Gal(β1-4)Glc (9.6% of total peak area), with latter two not being found in breast milk
298 (Urashima, Messer & Oftedal, 2017), despite the fact that neutral OS represent the highest
299 percentage of HMO (Kunz et al., 2000).

300 In addition, the sample was analysed by HILIC coupled to mass spectrometry for
301 structural assignment. This allowed the identification of 19 structures, ranging between 300

and 1200 Da. Five out of 19 structures (3'-fucosyllactose, 3-SL, 6-SL, 6'-sialyllactosamine and LNnT, which account for a total peak area of 56.52%) are also found in breast milk (Table 2). Furthermore, using the same analytical technique as Mariño et al. (2011), 4 structures were detected in the OSF, which were not reported in that study and these included: 3'-fucosyllactose, NeuAc(α2-3)Gal(β1-3)Gal(β1-4)Glc and the *O*-acelylated forms of two sialylated oligosaccharides (NeuAc(α2-3)Gal(β1-4)Glc and NeuAc(α2-8)NeuAc(α2-3)Gal(β1-4)Glc). Finally, only a small proportion (0.4% of total peak area) of Neu5Gc was detected in the OSF, which is particularly important for applications in infant formulas as Neu5Gc [present in numerous mammals, but not in humans (Varki & Marth, 1995)], is known to be antigenic (Varki & Schauer, 2009). To the best of our knowledge, this is the first time that such a well characterised SL-enriched powder, which also contains a variety of other OS structures, has been produced from commercial dairy streams.

3.2. Effect of BMO on interaction between respiratory cells and S. pneumoniae

To determine the concentration of the OSF that should be tested in the in vitro assays, a number of options were considered such as adding an amount of powder equivalent to the concentration of HMO (10–15 g L^{-1}). However, as the OSF is not a pure mixture of BMO, a concentration of BMO corresponding to physiological concentrations of 6-SL, which is a predominant acidic oligosaccharide in breast milk was selected. Furthermore, previous studies demonstrated the importance of the α2-6 linkage on interactions with bacteria (Kavanaugh et al., 2013; Marotta et al., 2014). Marotta et al. (2014) found that 6-SL inhibits *Pseudomonas aeruginosa* PAK invasion of pneumocytes. Kavanaugh et al. (2013) demonstrated that 6-SL increased adhesion of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 to HT-29. Consequently, 6-SL was chosen as an indicator of OS concentration and the

concentration of 6-SL in the OSF was matched to levels of 6-SL found in breast milk (0.1 to 0.3 mg mL^{-1}; Kunz et al., 2000) when tested on pharynx cells. In fact, this is the potential concentration that an infant's URT would be exposed to during regular breast feeding.

The OSF significantly reduced the adhesion of *S. pneumoniae* R6 to the pharynx cells Detroit 562 by 78%, 51% and 25% at OSF concentrations of 6 mg mL^{-1} ($P<0.001$), 4 mg mL^{-1} ($P<0.001$) and 2 mg mL^{-1} ($P<0.001$), respectively (Fig. 1). The data demonstrated that the anti-adhesion effect was concentration dependent with the largest anti-adhesive effect seen at the highest physiological concentration tested, 0.3 mg mL^{-1} (Fig. 1). This suggests that the application of such an OSF should involve the exposure of infants' URT to reported physiological range of HMO. In addition the effect of long term exposure to an OSF should also be considered.

The experiment was repeated testing 6 mg mL^{-1} solutions of demineralised whey and OSP, which are the initial and intermediate material prior to OSF production. Solubility issues meant that higher concentrations could not be tested. In both cases, a minimal (~5%) and not significant reduction of adhesion was observed (Fig. 2). These results suggest that the higher levels of OS present in the OSF were responsible for the observed effect. In fact, demineralised whey, OSP and OSF were tested using the same concentration (6 mg mL^{-1}) of powder, whereas SL concentration increased from 0.003 mg mL^{-1} in demineralised whey to 0.072 mg mL^{-1} in the OSP to a final 0.3 mg mL^{-1} in the OSF.

The effect of the OSF in reducing the adhesion of *S. pneumoniae* R6 to the lung cell line A549 was also investigated. In this case, concentrations of OSF in the range of 0.24 and 2.4 mg mL^{-1} were used, which correspond to 6-SL concentrations in the range of 0.0125–0.125 mg mL^{-1}. These concentrations were employed for similar studies on *P. aeruginosa* (Marotta et al., 2014) and represent the lowest and highest estimated concentration of the acidic fraction of HMO in infant blood (Bode et al., 2004b), which may potentially reach the

lungs. The adhesion of *S. pneumoniae* R6 was significantly reduced by 55, 34 and 17%, following pre-incubation with the OSF at a concentration of 2.4 mg mL^{-1} ($P < 0.001$), 1.15 mg mL^{-1} ($P < 0.001$) and 0.24 mg mL^{-1} ($P < 0.005$) (Fig. 3). As the powder was re-suspended in the required media and a control of media alone was included, the effect could be solely attributed to the OS and not to any component in the media.

The data reported above is in agreement with Barthelson et al. (1998). In that study, the authors concluded that *S. pneumoniae* relies to a significant extent upon sialylated oligosaccharide ligands terminating in NeuAcα2-6(or 3)Galβ1 for adherence to epithelial cells. The predominance of α2-6 and α2-3 sialylated oligosaccharides in the OSF, which could act as decoys of the natural receptors of *S. pneumoniae*, could explain the ability of the OSF in reducing *S. pneumoniae* adhesion to respiratory epithelial cells.

As the OSF significantly reduced the adhesion of *S. pneumoniae* R6 to the pharynx and lung cells, further studies were carried out to determine if that observed effect was due to the interaction of the OS with the bacteria or epithelial cells. To determine if OS interacted with bacteria, the assay was carried out as described, with the removal of unbound OS prior infection of respiratory epithelial cells. Following the removal of free OS, the adhesion of *S. pneumoniae* R6 to pharynx and lung cells was still significantly ($P < 0.001$) reduced by 77% and 48%, respectively (Fig. 4). To determine if the OS interacted with the respiratory epithelial cells, OSF was first incubated with the pharynx and lung cells. Following 30 min incubation, OS were removed and respiratory epithelial cells were infected with *S. pneumoniae* R6. No anti-adhesive effect was observed following this modification to the adhesion assay (Fig. 4).

The results would indicate that the ability of OS to reduce the adhesion of *S. pneumoniae* R6 to epithelial cells of the respiratory tract was mediated by interaction of OS with the bacteria and not with the epithelial cells, in agreement with results observed by

Marotta et al. (2014). Furthermore, the results demonstrate that the OSF was not cytotoxic to respiratory epithelial cells, since the adhesion of bacteria to respiratory epithelial cells alone and OSF exposed cells was comparable. Furthermore, the viability of the lung cells making up the confluent monolayer was determined with and without OSF before commencing the adhesion assays to ensure that OSF was not toxic to the A549 cells. The viability was approximately 90% ($P = 0.27$), demonstrating that the growth of the A549 cells was not affected by the exposure to OSF.

Taken together, the in vitro results reported in the present study suggest that BMO could be effective in protecting infants from upper and lower respiratory infections associated to *S. pneumoniae*. The precise mechanism of how *S. pneumoniae* establishes and maintains colonisation has yet to be fully characterised. It is clear, however, that the bacterium's glycosidases play a key role in colonisation, as these enzymes are capable of modifying *N*-linked glycans, *O*-linked glycans, and glycosaminoglycans on the host epithelial surface, thereby rendering the host susceptible to colonisation (Bogaert et al., 2004; Tong, Blue, James, & DeMaria, 2000). For instance, NanA cleaves α2-3- and α2-6-linked sialic acid, while NanB is specific to α2-3-linked sialic acid (Gut, King, & Walsh, 2008). Furthermore, BgaA the β-galactosidase is specific to galactose β1-4 linked to *N*-acetylglucosamine (Galβ1-4GlcNAc), commonly found in complex *N*-linked glycan structures (King, Hippe, & Weiser, 2006; Zähner & Hakenbeck, 2000; Zeleny, Altmann, & Praznik, 1997). It is the modification of the host epithelial surface by these glycosidases that is the first step in bacterial colonisation. As the OSF generated in this study is particularly rich in these structures, it is possible that the anti-adhesive function is due to a decoy effect as has been previously suggested in the literature (Hickey, 2012; Morrow, Ruiz-Palacios, Jiang, & Newburg, 2005; Newburg, 2000).

5. Conclusions

This study reports the extraction of BMO in gram quantities from whey, employing a combination of membrane filtration and size-exclusion chromatography. The final product was characterised not only by the presence of predominant sialyllactose, but also by many other sialylated and neutral structures. This product was demonstrated to reduce adhesion of *S. pneumoniae* to pharynx and lungs cells, when it was tested at different physiological concentrations. This study further supports the potential production of value-added ingredients from whey streams, which could be used as functional ingredients in infant formulas and, more broadly, in foods with health benefits.

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Figure legends

Fig. 1. Effect of concentration of the oligosaccharide-enriched fraction (OSF) on *S. pneumoniae* R6 adhesion to Detroit 562 pharynx cells; control (CNT) was *S. pneumoniae* R6 in the absence of saccharide. Data are means ± standard deviation of assays carried out on three separate occasions in triplicate; an asterisk indicates $P < 0.001$.

Fig. 2. Effect of different substrates on interaction of *S. pneumoniae* R6 on pharynx cells. *S. pneumoniae* R6 was incubated with 6 mg mL^{-1} of demineralised whey powder (DWP), oligosaccharide-enriched powder (OSP) and oligosaccharide-enriched fraction (OSF) in EMEM supplemented with 2% FBS (v/v); control (CNT) was performed with no saccharide. Data are means ± standard deviation of assays carried out in triplicate; an asterisk indicates $P < 0.001$.

Fig. 3. Effect of concentration of the OSF on *S. pneumoniae* R6 adhesion to A549 lung cells; control (CNT) was *S. pneumoniae* R6 in the absence of saccharide. Data are means ± Standard deviation of assays carried out on three separate occasions in triplicate; an asterisk indicates $P < 0.005$.

Fig. 4. Interaction of oligosaccharide-enriched fraction (OSF) with *S. pneumoniae* R6 (■) or with eukaryotic cells (■). Left-hand set of data: *S. pneumoniae* R6 was incubated with 6 mg mL^{-1} of OSF in EMEM supplemented with 2% FBS. After incubation the unbound oligosaccharides were removed before the bacteria were used to infect the pharynx cell (■). Pharynx cells were incubated with 6 mg mL^{-1} of OSF in the medium above. The unbound OSF was removed from the pharynx cells, which were subsequently infected with *S.*

pneumoniae R6 (■). An asterisk indicates $P < 0.001$. Control (□, pharynx cells) was performed with no saccharide. Right-hand set of data: *S. pneumoniae* R6 was incubated with 2.4 mg mL^{-1} of OSF in DMEM supplemented with 2% FBS. After incubation the unbound oligosaccharides were removed before the bacteria were used to infect the eukaryotic cells (■). Lung cells were incubated with 2.4 mg mL^{-1} OSF in the above medium. The unbound OSF was removed from the lung cells, which were subsequently infected with *S. pneumoniae* R6 (■). An asterisk indicates $P < 0.001$. Control (CNT, lung cells) was performed with no saccharide.

Table 1

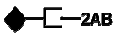
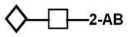
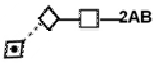
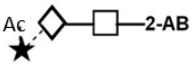
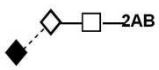
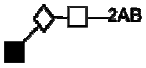
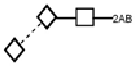
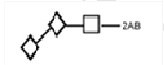
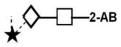
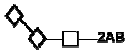
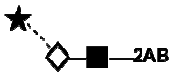
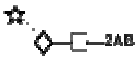
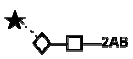
Enrichment of oligosaccharides from OSP employing size exclusion chromatography. [a]

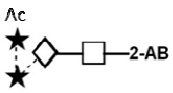
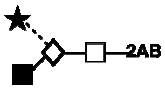
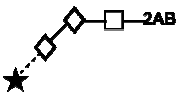
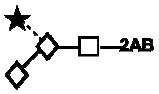
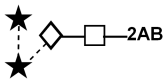
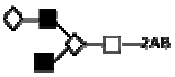
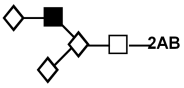
Oligosaccharide	OSP	OSF	Yield (%)
Lactose (g, total)	149.628	0.079	0.05
SL (g, total)	2.9175	2.084	71.43

[a] Abbreviations are: OSP, oligosaccharides-rich powder; OSF, oligosaccharides-rich fraction. Seven hundred and fifty millilitres of OSP were applied over 15 runs and 8.83 g of OSF were recovered pooling fractions from the 15 runs.

Table 2

Structural assignment of oligosaccharides in oligosaccharides-rich fraction (OSF).

Peak number	*m/z* observed	*m/z* theoretical	UXOF Symbol structural assignment	GU value	Relative % UPLC-HILIC-FLD
1	-	-	monosaccharides	1.00	-
2				1.03	-
3	502.22	502.20		1.88	0.7
	461.21	461.18			2.5
4				1.95	
5	-	-	neutral di- or tri-saccharides	2.28	-
6	-	-	acidic di- or tri-saccharides	2.33	0.1
7				2.36	0.02
				2.49	
8	607.27	607.24			0.7
	794.32	794.28			
9	664.29	664.26		2.71	23.8
10	664.29	664.26		2.83	1.3
11	623.26	623.23		2.89	1.3
					
12	623.26	623.23		2.98	9.6
			major component:		
13	752.31	752.27		3.15	
					47.8
	623.26	623.23	minor component:		
					
14	-	-	acidic tri- or tetrasaccharides	3.30	0.2
15				3.34	0.1
16				3.38	0.1
17	793.35	793.30		3.48	0.7
18	768.30	768.27		3.50	0.4
19	752.31	752.27			7.4
				3.56	
20	826.36	826.31		3.63	0.6

21	1085.47	1085.38		3.84	0.1
22	955.39	955.35		3.91	0.3
23	914.37	914.33		4.01	1.3
24	-	-	acidic oligosaccharide	4.11	0.03
25	914.37	914.33		4.33	0.2
26	1043.44	1043.37		4.58	0.4
27	1029.45	1029.39		4.69	-
28	988.43	988.36		4.84	0.1
29	-	-	acidic oligosaccharide	5.47	0.2

[a] Relative % UPLC-HILIC-FLD represents area of peaks compared with total peak area in HILIC chromatograms. Symbols are: ■, *N*-acetylglucosamine; □, glucose; ◇, galactose; ◆, *N*-acetylgalactosamine; ◈, fucose; ○, mannose; ★, *N*-acetylneuraminic acid; ✯, *N*-glycolylneuraminic acid; △, xylose. Linkages are denoted as: ---, α-linkage; —,β-linkage.

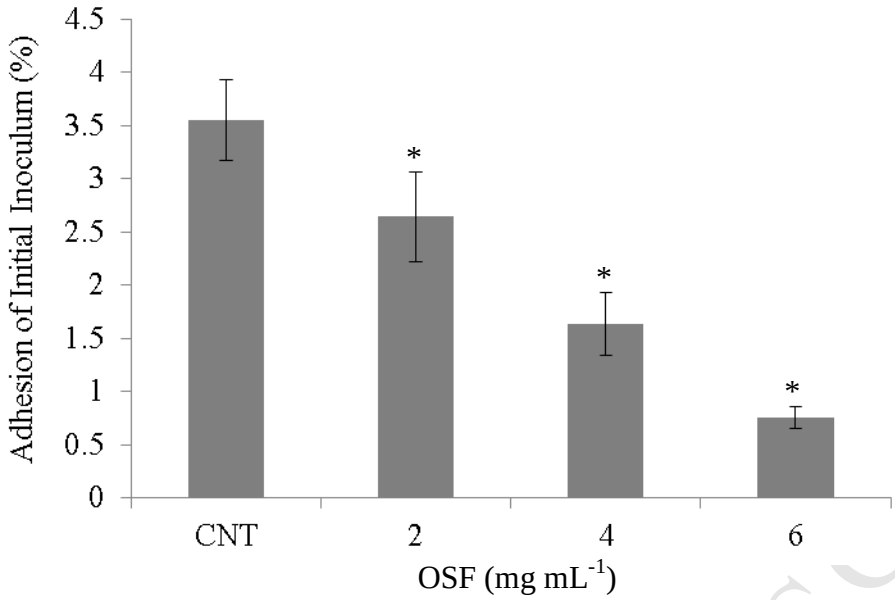


Figure 1.

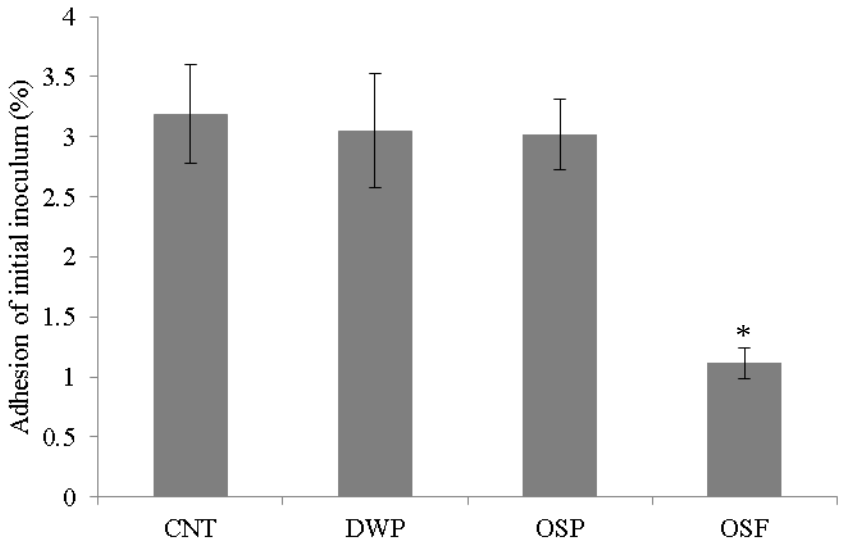


Figure 2.

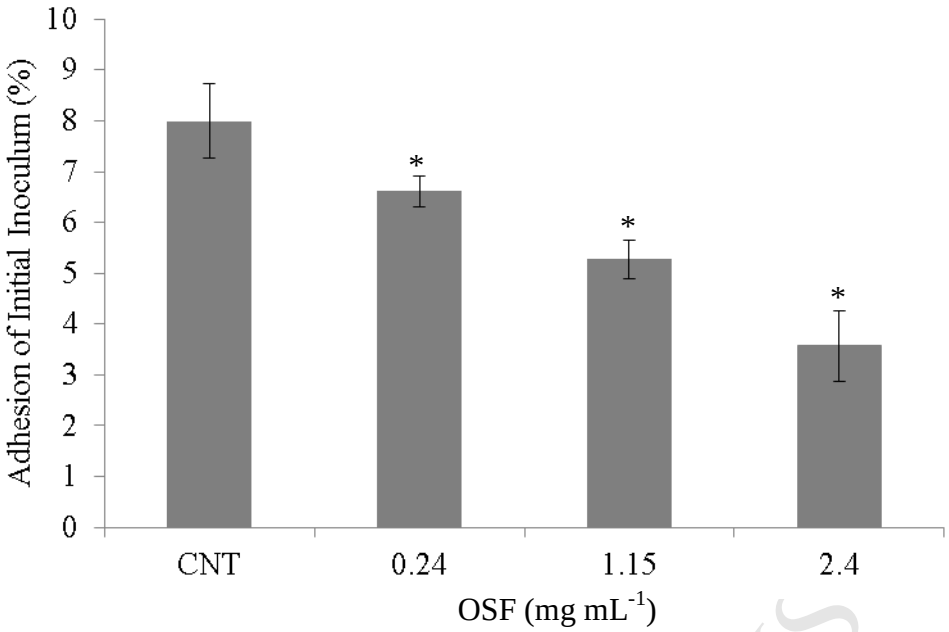


Figure 3.

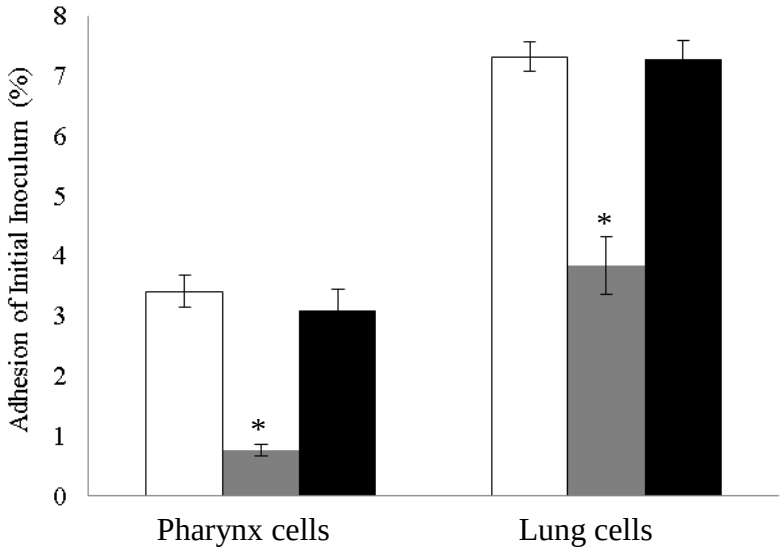


Figure 4.