



1-Amino-2'-fucosyllactose inhibits biofilm formation by *Streptococcus agalactiae*

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Abstract

2'-Fucosyllactose (2'-FL) is a ubiquitous oligosaccharide in human milk. Importantly, this carbohydrate promotes the growth of several strains of *Bifidobacteria*, a class of beneficial gut commensal, and inhibits epithelial binding of pathogens. In light of these protective effects, we elected to evaluate the potential of 2'-FL to serve as an antibacterial agent against Group B *Streptococcus* (GBS). While 2'-FL was devoid of any substantial antimicrobial or antibiofilm activity, conversion of 2'-FL to its reducing end β -amine provided a novel antibiofilm compound.

Biofilms consist of an organized population of bacteria that adhere to both abiotic and biotic surfaces. These bacteria are encapsulated by an extracellular polymeric matrix [1, 2]. At the molecular level, this matrix consists of an extracellular polymeric substance (EPS) composed primarily of DNA, polysaccharides, and an assortment of proteins. The EPS is critical to the structural integrity of biofilms, the ability of biofilms to adhere to different surfaces, and the ability of bacteria to adhere to and communicate with one another [3]. Biofilms are a major concern to human health as it is estimated that they are involved in upwards of 80% of microbial infections [1]. Indeed, the majority of bacterial pathogens use biofilm as a key virulence factor to enhance

their pathogenicity and enable their survival in a hostile host environment [4–6]. In particular, one hallmark feature of bacteria in the biofilm state is an increased resistance to antibiotics [1, 2, 7, 8]. Remarkably, these species are up to 1000-fold more resistant to antimicrobial agents than bacteria in the planktonic state [1, 8].

Protections uniquely afforded to bacterial cells by biofilms can be attributed to several features of the biofilm environment. Perhaps most obvious is the physical barrier formed by the EPS between bacterial cells and a hostile outside environment. This barrier serves to limit the penetration of antimicrobials as well as host defense mechanisms to all areas of the biofilm [2, 9]. While bacteria in the EPS are shielded from harsh external conditions, cells within the matrix encounter harsh internal growth conditions. For example, it has been shown that oxygen levels can be depleted by upwards of 30-fold at the center of large biofilm matrices [1, 10]. Additionally, nutrient availability is lowered in certain areas of the matrix [2, 7].

As a result of these harsh conditions, cells deep within the biofilm matrix exist in a slow-growing state. This decreased metabolic growth rate plays a key role in resistance evolution to antibiotics that target growth factors. For example, β -lactams are ineffective against bacterial cells that are not undergoing cell division [1, 2, 9]. Indeed, persistent cells (dormant versions of regularly growing cells) have increased resistance to antibiotics and can restore depleted biofilm communities after chemotherapy is concluded [9]. In sum, the ability of bacteria in a biofilm matrix to adapt to a sessile lifestyle by undergoing phenotypic shifts in metabolic rates and gene expression is an important

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aspect of biofilm as a resistance mechanism. Accordingly, the development of antibiofilm agents are as important to human health as the development of new antibiotics.

In an effort to discover novel molecular scaffolds, our team recently embarked on a program aimed at assessing the antimicrobial and antibiofilm properties of human milk oligosaccharides (HMOs). Initial studies revealed that HMOs govern bacterial growth and biofilm assembly [11–15]. We discovered that HMO extracts possess antibiofilm and antimicrobial properties against *Streptococcus agalactiae* (GBS), antimicrobial properties against the Gram-negative aerobe *Acinetobacter baumannii*, and antibiofilm properties against methicillin-resistant *Staphylococcus aureus* (MRSA). In a second-generation study, we observed that HMOs potentiate the actions of aminoglycosides, macrolides, lincosamides, and tetracyclines against GBS. However, HMOs did not potentiate the actions of β -lactams or glycopeptides—antibiotics that inhibit cell wall synthesis [15]. HMOs also potentiated the action of aminoglycosides against both *S. aureus* and *A. baumannii*. Given these patterns, we hypothesized the mode of action behind HMO-mediated activity potentiation was an increase in cellular permeability. This hypothesis was tested and validated using a membrane permeability assay [15]. In addition to probing the mechanism of antimicrobial action, we initiated a series of studies designed to interrogate the activity of structurally-defined, homogenous HMOs [13, 14]. These studies have provided key insights into the structural features required for activity (sialylation vs fucosylation).

The contribution described herein is an extension of these studies as it concerns the potential of a ubiquitous HMO, 2'-fucosyllactose **2** (Fig. 1), to serve as an antibacterial agent against GBS. 2'-fucosyllactose (2'-FL), a monofucosylated derivative of lactose **1** wherein fucose is linked via an α -1,2 linkage to the terminal galactose residue, is generally considered the most abundant oligosaccharide as 70–80% of milk samples contain this HMO [16]. Due to its ease of synthesis, both chemically and enzymatically, 2'-FL is also the most studied HMO [17]. As is characteristic of all HMOs, 2'-FL is not metabolized by digestive enzymes. Once in the colon, it serves a prebiotic fiber by providing a selective growth advantage to beneficial gut microbes. In this regard, 2'-FL plays an important role in gut development [17, 18]. 2'-FL has also been shown to enhance immune system development, prevent the formation of

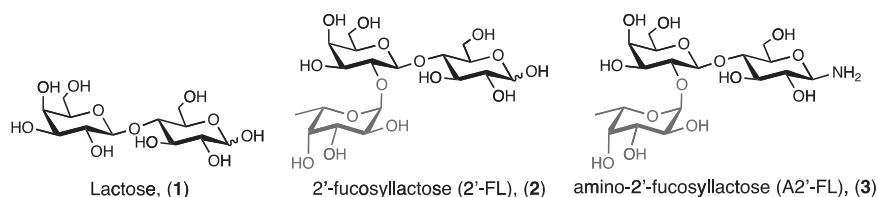
allergies, prevent pathogen adhesion to the epithelium, and assist in development of cognition and brain function. Due to its numerous neonatal health benefits, 2'-FL has become a common additive in infant formula; infant formula is typically composed primarily of bovine milk which lacks the complex oligosaccharides found in human milk.

We initiated studies to evaluate the antimicrobial and antibiofilm properties of 2'-FL against GBS. As previous work from our laboratory has showed that the antibacterial properties of heterogenous HMOs are strain-specific, we chose to screen 2'-FL against two different strains of GBS (GB590 and GB2) to determine whether the molecule possessed strain-specific activity. Importantly, GB590 and GB2 are different serotypes. Ten GBS serotypes exist (Ia, Ib, II-IX) with each serotype being defined by the structure of its capsular polysaccharides (CPS) [19, 20]. GB590 is a serotype III strain; serotype III strains account for the highest number of GBS infections [19]. GB2 is a serotype Ia strain; serotype Ia is one of the five serotypes responsible for more than 85% of the global burden of GBS disease [11, 12, 19].

To assess antimicrobial activity, GBS growth and viability in Todd-Hewitt Broth (THB) alone or in THB dosed with ca. 5 mg/mL of either 2'-FL or lactose was monitored over 24 h. Although lactose itself is not an HMO, it does serve as the core structure for 2'-FL (Fig. 1). Thus, inclusion of lactose in the analysis would allow us to determine whether the fucose residue of 2'-FL was necessary for antibacterial activity. Growth was quantified via spectrophotometric readings at OD₆₀₀ while viability was quantified via serial dilution of bacterial cultures and plating onto blood agar plates with subsequent enumeration of colony forming units (CFUs) the following day. We screened 2'-FL at 5 mg/mL as we previously determined that the IC₅₀ values of heterogenous HMO mixtures against both GB590 and GB2 were ca. 5 mg/mL. Thus, we hypothesized that dosing 2'-FL at this concentration would allow for observation of any antimicrobial activity without completely preventing bacterial growth. Moreover, this concentration represents the low end of physiological concentrations (5–25 mg/mL).

We observed that 2'-FL did not possess antimicrobial activity against either GBS strain (Figs. 2 and 3). Against GB590, 2'-FL showcased modest growth reduction compared to bacteria grown in THB at 4 and 24 h. At 4 h, growth was decreased by ca. 20% while at 24 h it was

Fig. 1 Structures of lactose, 2'-fucosyllactose (2'-FL), and amino-2'-fucosyl lactose (A2'-FL). Lactose is shown in black, fucose is highlighted in red



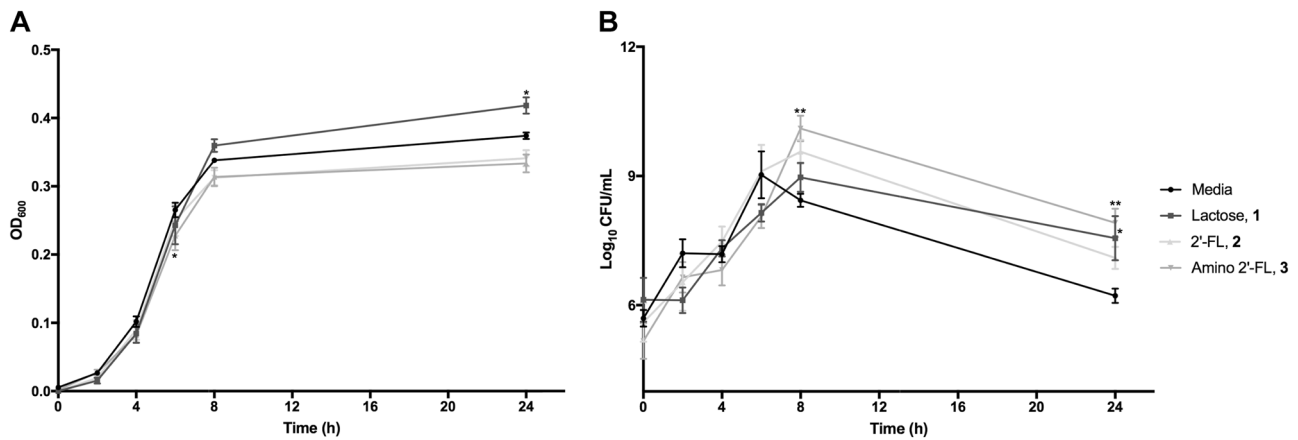


Fig. 2 Effects lactose (1), 2'-FL (2), and amino 2'-FL (3) at ca. 5 mg/mL on the growth and viability of GB590 in THB (a) OD₆₀₀ readings were recorded at 0, 2, 4, 6, 8, and 24 h. Average OD₆₀₀ for each carbohydrate and time point is marked with the corresponding symbols. (b) Enumeration of CFU/mL was performed at 0, 2, 4, 6, 8, and 24 h corresponding to the OD values graphed in chart (a). Mean log₁₀ CFU/mL for each carbohydrate and time point is marked with the

corresponding symbols. Data displayed represent the mean OD₆₀₀ or log₁₀CFU/mL ± SEM of at least three autonomous experiments, each with 3 technical replicates. * represents $p = 0.0142$ in (a), and * and ** represent $p = 0.0136$ and $p = 0.0051$, respectively in (b) by two-way ANOVA with *posthoc* Dunnett's multiple comparison test comparing the growth and viability of GB590 in each supplemented condition to the growth and viability of GB590 in media alone

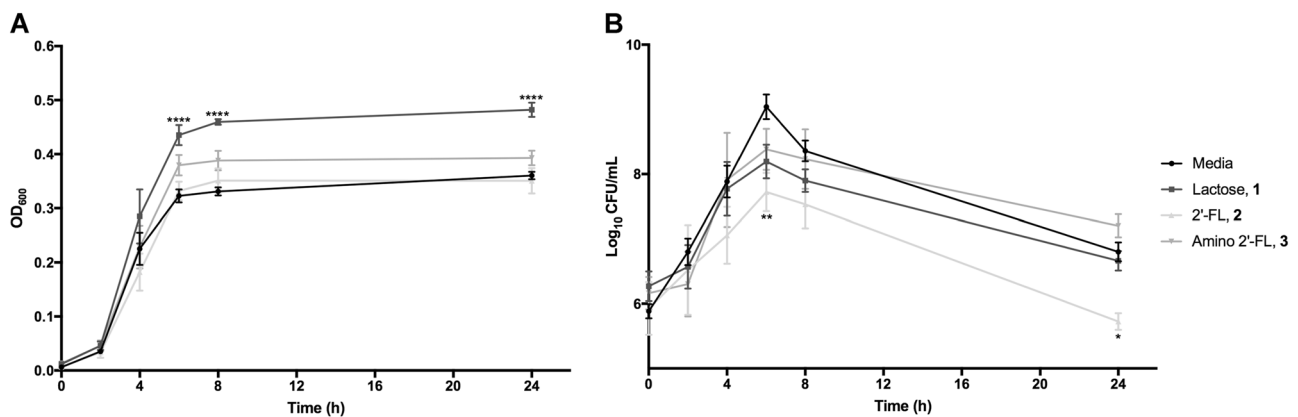


Fig. 3 Effects of lactose (1), 2'-FL (2), and amino 2'-FL (3) at ca. 5 mg/mL on the growth and viability of GB2 in THB (a) OD₆₀₀ readings were taken at 0, 2, 4, 6, 8, and 24 h. Average OD₆₀₀ for each carbohydrate and time point is marked with the corresponding symbols. (b) Enumeration of CFU/mL was performed at 0, 2, 4, 6, 8, and 24 h corresponding to the OD values graphed in (a). Average log₁₀ CFU/mL for each carbohydrate and time point is marked with the

corresponding symbols. Data displayed represent the average OD₆₀₀ or log₁₀CFU/mL ± SEM of at least three autonomous experiments, each with 3 technical replicates. **** represents $p < 0.0001$ in (a), and * and ** represent $p = 0.0146$, $p = 0.00077$, respectively in (b) by two-way ANOVA with *posthoc* Dunnett's multiple comparison test comparing the growth and viability of GB2 in each supplemented condition to the growth and viability of GB2 in media alone

reduced by approximately 11%. Despite minor decreases in bacterial growth, 2'-FL treatment had no effect on GB590 viability at any point in the 24 h time frame (Fig. 2). Alternatively, while treatment with 2'-FL did not alter GB2 growth, it did result in a modest reduction (15%) in viability at both 6 and 24 h (Fig. 3).

While the lack of antimicrobial activity was initially surprising, the extensive literature detailing the protective effects of 2'-FL against pathogenic colonization offers an explanation for this lack of activity. While 2'-FL protects infants from infection, this protection arises from its ability to serve as an antiadhesive antimicrobial agent. 2'-FL is a

soluble decoy receptor that competes with epithelial cell surface glycans for binding. Pathogen binding to epithelial cells is the first step in the infection process [18].

For instance, 2'-FL inhibits *Campylobacter jejuni* binding to intestinal cells as well as the binding of noroviruses to histo-blood group antigens (HBGAs) [21–24]. 2'-FL also inhibits *Pseudomonas aeruginosa* adhesion to epithelial cells [25, 26]. These results, in combination with our study, suggest that the environment wherein 2'-FL encounters a pathogen is as important to this HMO's antimicrobial properties as its molecular structure. Thus, we hypothesize that any antimicrobial activity of 2'-FL against GBS would

likely similarly be attributable to an ability to prevent bacterial adhesion to epithelial cells. This mechanism of action would not, however, have been assessable with the assays employed in this study.

In addition to evaluating antimicrobial activity, we investigated the ability of 2'-FL to alter biofilm production. Biofilm production was evaluated using a plate-based assay after 24 h of growth. This assay permits quantification of growth using spectrophotometric evaluation at OD₆₀₀ followed by crystal violet staining of any adherent bacteria with subsequent spectrophotometric evaluation at OD₅₆₀ to quantify biofilm levels. To account for any antimicrobial activity, results are expressed as a ratio of biofilm produced to bacterial cells present. Biofilm production for GB590 and GB2 when treated with 2'-FL relative to biofilm formation when grown in media alone is shown in Fig. 4. Once again, 2'-FL was devoid of activity.

Although 2'-FL itself failed to alter GBS biofilm production, we hypothesized that we could convert 2'-FL to an antibiofilm compound by incorporating a positive charge into the structure. This hypothesis was founded on the premise that biofilm matrices are largely composed of anionic polymeric substances and negatively charged extracellular DNA. Indeed, cationic molecules disrupt biofilm matrices [5, 27–36]. The most facile method to incorporate a positive charge into the structure of 2'-FL (or the reducing end of any saccharide) is to convert the anomeric alcohol to an amine using the Kochetkov amination [37]. In the event, 2'-FL was exposed to ammonium carbonate and

heated for 72 h to generate amine **3** in near quantitative yield as the β -anomer (Scheme 1).

Much to our delight, while the effects of amino 2'-FL derivative **3** on GB590 and GB2 growth and viability were generally comparable to those of 2'-FL (Figs. 2 and 3), compound **3** was a significantly more effective antibiofilm compound than 2'-FL (Fig. 4). Impressively, exposure of GB590 and GB2 to ca. 5 mg/mL of **3** resulted in an average decrease in biofilm production of 37% for GB590 and 46% for GB2 compared to the control. It is important to note that compound **3** does not decompose under the assay conditions.

In summation, we have discovered that while 2'-FL is largely devoid of antimicrobial activity against GBS and possesses no antibiofilm activity against this pathogen, conversion of 2'-FL to an anomeric, amino-variant produced a compound that showcased impressive antibiofilm activity against GBS. While the mechanism of action behind this antibiofilm activity remains unknown, we hypothesize the positively-charged carbohydrate could serve as a surfactant. Assays to investigate this potential mechanism of action are ongoing. Furthermore, while 2'-FL did not showcase antimicrobial activity against GBS in this study, we hypothesize that a more judicious choice of assay will reveal the potential antiadhesive antimicrobial actions of 2'-FL against GBS. Future studies in this regard will use association assays to assess levels of GBS attachment to epithelial cells when GBS is challenged with 2'-FL. Results of these studies will be reported in due course.

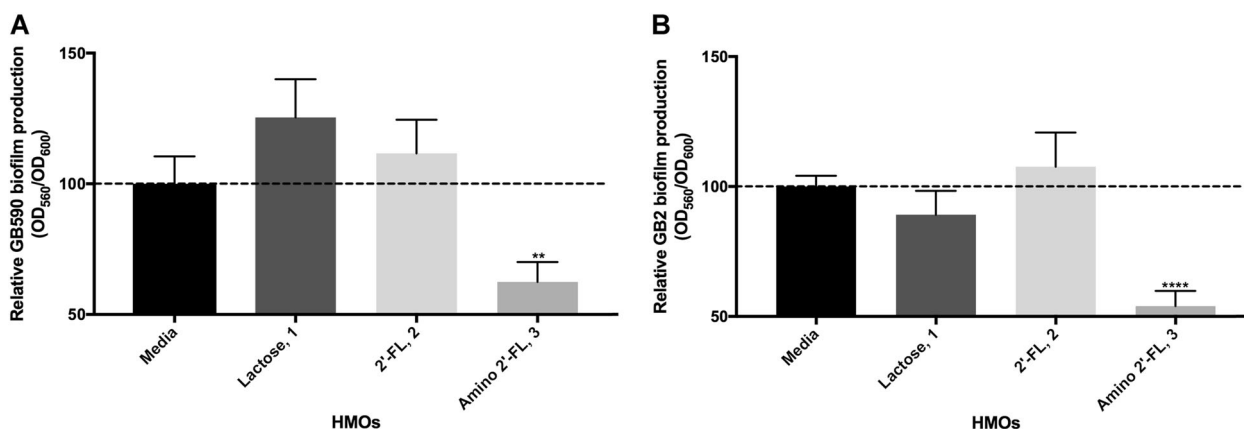
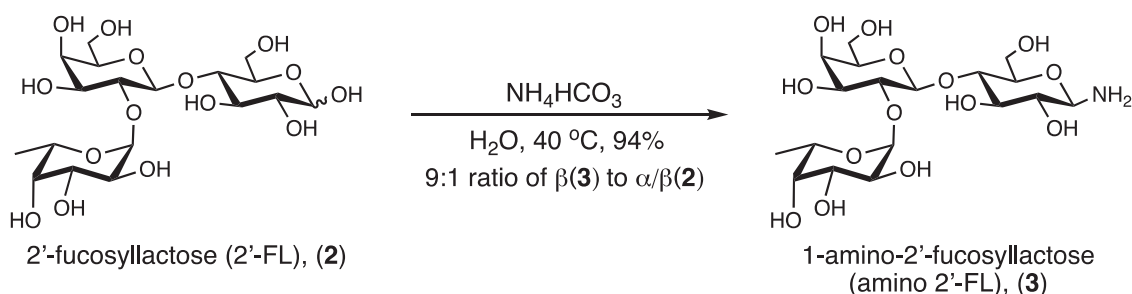


Fig. 4 Effects of lactose (**1**), 2'-FL (**2**), amino 2'-FL (**3**) at ca. 5 mg/mL on GBS biofilm production after 24 h. **a** Biofilm levels, denoted by the ratio of biofilm/biomass (OD₅₆₀/OD₆₀₀), by GB590 in the presence of carbohydrates relative to biofilm levels in THB alone. The data represent the relative average biofilm/biomass ratio \pm SEM of three autonomous experiments, each with 3 technical replicates, where biofilm production of GB590 in media alone is assigned a value of 100%. ** represents $p = 0.0094$ by one-way ANOVA, $F = 7.562$ with posthoc Dunnett's multiple comparison test comparing biofilm production of GB590 in each carbohydrate supplementation condition to biofilm production of GB590 in THB alone. **b** Biofilm levels, denoted

by the ratio of biofilm/biomass (OD₅₆₀/OD₆₀₀), by GB2 in the presence of carbohydrates relative to biofilm levels in THB alone. The data represent the relative average biofilm/biomass ratio \pm SEM of at least three autonomous experiments, each with 3 technical replicates, where biofilm production of GB2 in media alone is assigned a value of 100%. **** represents $p < 0.0001$ by one-way ANOVA, $F = 9.961$ with posthoc Dunnett's multiple comparison test comparing biofilm production of GB2 in each supplemented condition to biofilm production of GB2 in THB alone. Mean GBS biofilm levels in media are marked with a dotted line



Scheme 1 Synthesis of 1-amino-2'-fucosyllactose (amino 2'-FL), (3)

Materials and methods

Synthesis

2'-FL (488 mg, 1.0 mmol) was dissolved in water (10 mL) and $(\text{NH}_4)\text{HCO}_3$ (790 mg, 10.0 mmol) was added. The resultant slurry was warmed to 40 °C and stirred for three days. The clear supernatant was filtered through a plug of cotton, frozen, and lyophilized. The lyophilization was determined to be complete when the mass of the product remained constant. The glycosyl amine was obtained as a white solid (458 mg, 0.94 mmol, 94%, 9:1 ratio of product to starting material): R_f 0.15 (60:30:3:5 CHCl_3 : CH_3OH : AcOH : H_2O); ^1H NMR (400 MHz, D_2O) δ 5.19 (d, $J = 2.2$ Hz, 1 H, H_1''), 4.55 (dd, $J = 2.6, 8.5$ Hz, 1 H, H_1), 4.40 (d, $J = 7.7$ Hz, 1 H, H_1'), 4.18–4.06 (m, 1 H), 3.90–3.14 (m, 15 H), 1.12 (t, $J = 7.4$ Hz, 3 H); ^{13}C NMR (100 MHz, D_2O) δ 100.1, 99.2, 84.9, 82.9, 76.1, 75.9, 75.1, 74.9, 73.9, 73.5, 71.5, 69.5, 69.0, 68.0, 66.76, 61.0, 60.2, 15.2; Ultraflex TOF MS in reflectron positive mode ($\text{M} + \text{Na}$) $^+$ calcd for $\text{C}_{18}\text{H}_{32}\text{NNaO}_{14}$ 510.179, found 510.170.

Materials

2'-fucosyllactose was kindly donated by Glycosyn and FrieslandCampina. D-lactose monohydrate was purchased from Sigma Aldrich.

Microbiology

Bacterial strains and culture conditions, bacterial growth and viability assays, bacterial biofilm assay, and statistical analysis were completed as previously described [11–15].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict to declare.

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