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Anti-biofilm activity of human milk oligosaccharides against multi-drug resistant and susceptible isolates of *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii is a serious threat to human health, per the Centers for Disease Control and Prevention's latest threat assessment. *A. baumannii* is a gram-negative opportunistic bacterial pathogen that causes severe community and nosocomial infections in immunocompromised

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Supporting Information

These data are available as supporting files in "Supporting Information". This information is available free of charge on the ACS Publications website.

Ethical Considerations

Ethics approval to carry out this study was provided by the Meharry Medical College Institutional Review Board (IRB 081204AAH23119) and by Vanderbilt University Medical Center (IRB 100897). The secondary use of deidentified or coded samples is not considered research involving human subjects under 45 CFR 46. Biospecimens used in this study were deidentified and need for consent was waived by the IRB in accordance with federal regulation (45 CFR 46, Department of Health and Human Services, Authority: 5 U.S.C. 301; 42 U.S.C. 289(a); 42 U.S.C. 300v-1(b)).

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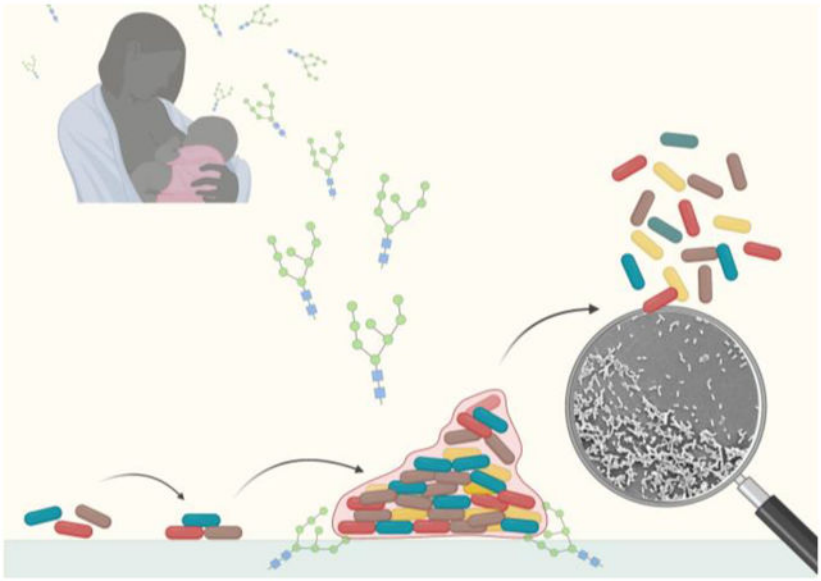
patients. Treatment of these infections is confounded by the emergence of multi- and pan-drug resistant strains of *A. baumannii*. *A. baumannii* colonizes abiotic and biotic surfaces and evades antimicrobial challenges by forming biofilms, which are three-dimensional architectural structures of cells adhered to a substrate and encased in an extracellular matrix comprised of polymeric substances such as polysaccharides, proteins, and DNA. Biofilm-inhibiting compounds have recently gained attention as a chemotherapeutic strategy to prevent or disperse *A. baumannii* biofilms and restore the utility of traditional antimicrobial strategies. Recent work indicates that human milk oligosaccharides (HMOs) have potent antibacterial and biofilm-inhibiting properties. We sought to test the utility of HMOs against a bank of clinical isolates of *A. baumannii* to ascertain changes in bacterial growth or biofilm formation. Our results indicate that out of 18 strains tested, 14 were susceptible to the anti-biofilm activities of HMOs, and that the potent anti-biofilm activity was observed in strains isolated from diverse anatomical sites, disease manifestations, and across antibiotic-resistant and susceptible strains.

Graphical Abstract



Keywords

antimicrobial; biofilm; glycobiology; innate immunity; *Acinetobacter*

Acinetobacter baumannii is a gram-negative coccobacillus bacterium that exhibits multi-drug resistance, causes severe infections and is classified as an urgent global health threat[1]. Particularly known for its survival on equipment in hospital settings, most *A. baumannii* infections are nosocomially acquired, usually in intensive care units where patients are already immunocompromised[2]. In recent years, *A. baumannii* infections have increased in frequency among military personnel in the Middle East, specifically Iraqi combat zones[3]. *A. baumannii* is responsible for a wide variety of infections across various anatomical sites within the body including: urinary tract infections, pneumonia, sepsis, and dermal infections. Of those, one of the most common manifestations is ventilator acquired pneumonia (VAP)[4].

A. baumannii infections in intubated patients have a 25% mortality rate while patients requiring vasopressors experience a greater than 50% mortality rate[5]. Concerningly, a recent molecular survey of the microbiota of intubated patients in Nashville, Tennessee indicated *A. baumannii* could be detected in 100% of surveyed patient tracheal tissue samples post-intubation, a result that was not observed in healthy controls[6].

With increasing multi- and pan-drug resistance, *A. baumannii* presents a unique challenge to clinicians. It has been previously reported that *A. baumannii* rapidly develops resistance to antimicrobials and as such has been declared among the most serious ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) by the World Health Organization[7,8]. The dynamic deployment of several resistance mechanisms, including β-lactamases, efflux pumps, and target site modifications, has steadily decreased the availability of effective treatments against *A. baumannii*[9]. Additionally, *A. baumannii* has an impressive ability to adhere to and survive on surfaces in cell conglomerations otherwise known as biofilms.

Bacterial biofilms are microbial cell aggregates encased in a self-produced polymeric extracellular matrix. These cell communities adhere to both biotic and abiotic surfaces and manifest antimicrobial, host immune, and environmental defense mechanisms that are otherwise not present in planktonic cells[10]. The extracellular matrix of the biofilm helps bacterial cells evade antimicrobial molecules by sophisticated mechanisms including decreased diffusion of antimicrobial agents across the matrix and the deactivation of charged antibiotics via surface anionic molecules, among others.[2] However, numerous studies have also demonstrated that antibiotics can penetrate biofilms.[11–16] Chronic bacterial infections are often caused by biofilm-forming pathogens, and as such require prolonged antibiotic treatment at higher doses than needed for primarily planktonic cell infections[10]. This inevitably results in higher instances of antimicrobial tolerance which likely leads to increased morbidity and prolonged hospital stays. Antimicrobial tolerance, a phenomenon that has often been overlooked in clinic, has been shown to accelerate the development of antimicrobial resistance, which is an established global health threat that is often conferred in bacterial strains through the formation of biofilms[17]. Due to the imminent threat the antimicrobial resistance crisis has caused, disruption of bacterial behaviors that promote resistance mechanisms are of great interest. One way to achieve this is by employing adjuvant therapies, wherein molecules that regulate biofilm formation could be employed alongside antibiotics to weaken bacterial defenses[17]. These non-bactericidal therapies present a desirable mechanism as they do not exert evolutionary pressure on the bacterial population and thus do not promote further development of antimicrobial resistance.

Our work has centered around the exploration of human milk oligosaccharides (HMOs) as novel molecules to employ against antibiotic resistant pathogens. These molecules represent a complex group of sugars found in high concentrations in human breast milk and are known to possess prebiotic, antimicrobial, and antiadhesive properties[18]. Structurally composed of five monosaccharide units, HMOs have antibacterial activity against both gram-positive and gram-negative pathogens[19]. We have previously demonstrated the bactericidal power and anti-biofilm activity of HMOs in a variety of bacterial pathogens[20,21]. While our previous

work indicated HMOs did not have potent bactericidal activities against a single laboratory strain of *A. baumannii*, here we present our further investigations into the antibiofilm properties of these molecules, which have broad activity against a range of clinical strains of *A. baumannii*[19].

Results

Human milk contains a heterogeneous mixture of oligosaccharides

Human oligosaccharides were purified from 5 donors by size-exclusion chromatography and the identity of the oligosaccharides was determined by mass spectrometry techniques (Figure S1). Comparison with mass spectrometry profiles collected from oligosaccharide standards (Figure S2) revealed that human milk contains a heterogeneous mixture of 3-fucosyllactose (3-FL), 2'-fucosyllactose (2'-FL), difucosyllactose (DFL), lacto-N-fucopentasose I (LNFP I), lacto-N-fucopentasose II (LNFP II), lacto-N-fucopentasose III (LNFP III), lacto-N-triose II (LNT II), lacto-N-neohexaose (LNnH), para-lacto-N-neohexaose (para-LNnH), and lacto-N-tetraose (LNT), lacto-N-neotetraose(LNnT) (Table S1).

Human milk oligosaccharides do not repress *A. baumannii* growth or viability

Our previous work revealed that some HMOs received from a diverse group of donors had modest growth inhibition properties against the type strain, *A. baumannii* 19606T[22]. To study this further, we pooled purified HMOs from five donors and utilized these to study their collective effect on an expanded bank of clinical *A. baumannii* isolates. Nonetheless, exposure to increasing concentrations of HMOs (0, 0.5, 1.25, 2.5, 5.0 mg/mL) did not significantly inhibit the growth of the laboratory strain of *A. baumannii* 17978 or any other clinical isolates used in this study, with the notable exception of strain 17 which exhibited significant growth inhibition when cultured in the presence of 0.625 mg/mL of HMOs or higher (Figures S3, S4, S5, S6, and Table 1). Additionally, analysis of bacterial viability by quantitative culture indicates HMOs do not exert significant effects on bacterial viability across the clinical isolates of *A. baumannii* although a single strain (strain 17) exhibited a 1.2-log decrease in viability in the presence of HMOs, this was not statistically significant (Figure S7).

Human milk oligosaccharides repress biofilm formation of *A. baumannii* 17978

The laboratory strain of *A. baumannii* (17978) was utilized to analyze the effect of HMOs on bacterial biofilm formation. Exposure to 2.5 mg/mL of HMOs (an amount that does not affect bacterial growth and is physiologically relevant to the concentrations that would be found in human breast milk) resulted in an 8-fold decrease in bacterial biofilm formation as determined by colorimetric assay (Figure 1, panel A), a result that was statistically significant (P=0.0273, Student's *t* test). Furthermore, analysis by high-resolution electron microscopy (Figure 1, panel B) reveals drastic reduction in cellular adherence to plastic coverslips and tertiary architectural structures of cells indicative of biofilm formation.

Human milk oligosaccharides repress biofilm formation by sputum isolates of *A. baumannii*

A strain bank of *A. baumannii* clinical isolates from Nashville General Hospital in Nashville, Tennessee was previously assessed for biofilm formation patterns across anatomical isolation sites. Our prior work classified these isolates as a range of high and low biofilm formers as well as multi-drug resistant and susceptible isolates[2]. Of the seven sputum isolates tested, five showed a significant reduction in biofilm formation when supplemented with HMOs (Figure 2). Of the five isolates that showed a decrease in biofilm formation, three isolates formed substantial biofilms. Isolate 35, for instance, had a 15-fold decrease in biofilm formation following treatment with HMOs relative to the untreated control (P=0.0256; Student's *t* test and P<0.05; One-way ANOVA with Tukey's *post hoc* test). Similarly, the two other high biofilm-formers, isolates 38 and 79, showed a 4-fold reduction in biofilm formation in the presence of HMOs (P=0.0015 and P=0.0109, respectively; Student's *t* test, isolate 79 P<0.05; One-way ANOVA with Tukey's *post hoc* test). Moderate biofilm-formers, isolates 4 and 31, also underwent a 2-fold decrease in biofilm formation compared with the untreated control samples for each isolate (P=0.0043 and P=0.0278, respectively; Student's *t* test, strain 31 P<0.0001; One-way ANOVA with Tukey's *post hoc* test). By contrast, the remaining two isolates, 16 and 112, were low biofilm-formers and as such, no biofilm disruption was observed following HMO treatment relative to the untreated controls.

Human milk oligosaccharides repress biofilm formation by wound isolates of *A. baumannii*

Significant biofilm reduction was observed across all four wound isolates when supplemented with HMOs (Figure 3). Of the four isolates, three were high biofilm-formers that displayed a greater reduction in biofilm formation than the low biofilm-forming isolate 19. Among these, isolate 101 showed the largest difference with a 6-fold reduction in biofilm formation when treated with HMOs (P=0.0247; Student's *t* test, and P<0.01, One-way ANOVA with Tukey's *post hoc* test). Isolates 81 and 37 also had impressive biofilm reduction in the presence of HMOs with 5- and 4-fold reductions (P=0.0323 and P=0.0120, respectively; Student's *t* test, isolate 81 P<0.05; One-way ANOVA with Tukey's *post hoc* test). Interestingly, the low biofilm-forming isolate 19 also experienced a ~2-fold decrease in biofilm production following treatment (P=0.0228; Student's *t* test).

Human milk oligosaccharides repress biofilm formation by blood isolates of *A. baumannii*

A. baumannii blood isolates are considered among the highest biofilm-forming isolates in our collection[2]. We observed a significant reduction in biofilm formation among two of the three blood isolates in the presence of HMOs relative to the untreated control for each isolate (Figure 4). For instance, a 6-fold decrease in biofilm formation was observed in isolate 15 (P=0.0044; Student's *t* test, P<0.01; One-way ANOVA with Tukey's *post hoc* test), while biofilm formation in isolate 13 was reduced 5-fold in the presence of HMOs (P=0.0517; Student's *t* test). Isolate 17 also showed a moderate but significant 2-fold reduction in biofilm formation (P=0.0040; Student's *t* test).

Human milk oligosaccharides repress biofilm formation by urinary tract and abdominal isolates of *A. baumannii*

Urinary tract and abdominal isolates also displayed reduced biofilm formation in the presence of HMOs (Figure 5). Here, two out of three isolates showed significant reductions in biofilm formation, with two of the three isolates classified as high biofilm-formers. The high biofilm-forming isolate 11, for example, had a more than 5-fold reduction in biofilm formation in the presence of HMOs compared to the untreated control ($P=0.0277$; Student's *t* test, $P<0.05$; One-way ANOVA with Tukey's *post hoc* test). Additionally, isolates 5 and 32 had 2-fold reductions when treated with HMOs ($P=0.0020$ and $P=0.1939$, respectively; Student's *t* test).

Human milk oligosaccharides have broad anti-biofilm activity against *A. baumannii* clinical isolates of diverse anatomical origin

Analysis of grouped effect of HMOs on strains based on anatomical site of isolation (Figure 6) reveals that HMO treatment resulted in a 4-fold reduction in mean biofilm production in wound isolates ($P<0.0001$; Student's *t* test, $P<0.05$; One-way ANOVA with Tukey's *post hoc* test), a 3.7-fold reduction in sputum isolates ($P<0.0025$; Student's *t* test, $P<0.01$; One-way ANOVA with Tukey's *post hoc* test), a 5-fold reduction in blood isolates ($P<0.01$; One-way ANOVA with Tukey's *post hoc* test), and a 3.5-fold reduction in abdominal and urinary tract isolates ($P<0.0058$; Student's *t* test).

To confirm that HMOs can inhibit the number of viable cells adherent to a polystyrene surface, bacterial biofilms were grown in medium alone or supplemented with HMOs overnight as described above, then the following day biofilms were washed thoroughly to remove any planktonic cells. Biofilm cells were scraped into fresh medium and subjected to quantitative culture techniques to evaluate colony forming units per mL. The results in Figure S8 indicate that HMO treatment results in a 1-log and 0.9-log reduction in viable, adherent cells within the biofilms formed by *A. baumannii* 17978 and isolate 4, respectively ($P<0.05$; Mann-Whitney U test, n=3). Taken together, these results indicate that, in aggregate, HMOs are effective anti-biofilm compounds against clinical isolates of *A. baumannii* regardless of the source.

Human milk oligosaccharides can repress *A. baumannii* pellicle formation

Because *A. baumannii* biofilm has also been shown to form at the liquid air interface of a static culture, a structure referred to as "pellicle",[23] we sought to determine if HMOs could impact this phenotype. Bacterial pellicle formation was assessed by macroscopic examination of *A. baumannii* strains cultured in medium alone or medium supplemented with 2.5 mg/mL of HMOs in 12-well culture plates. Pellicle formation was inhibited by exposure to HMOs in strains 4, 31, 38, 79, 112, 19, 37, 81, 101, 13, 5, 11, and 32 (Figure S9), representing 76.5% of the assessed clinical isolates. Interestingly, strains 112, 13, and 32 did not exhibit significant differences in quantifiable biofilm in the presence or absence of HMOs, however, these three strains did form pellicles which were inhibited by exposure to HMOs.

Human milk oligosaccharides do not disrupt existing *A. baumannii* biofilms

Because HMOs have potent activity inhibiting *A. baumannii* biofilms, we sought to determine if HMOs could disrupt existing biofilms. To test this, we selected five strains that were susceptible to biofilm inhibition by HMOs (strains 4, 37, 38, 101, and 112) and inoculated static cultures (without HMO treatment) and allowed biofilms to form for 24 hours. The following day, media was gently removed from bacterial cells as to not disturb any biofilm formed and either fresh media alone or supplemented with 2.5 mg/mL of HMOs were added to the cultures for an additional 24 hours. Biofilm was quantified as previously described using the colorimetric assay. The results indicate that established biofilms are not significantly impacted by exposure to HMOs (Figure S10), underscoring that their anti-biofilm activity is likely targeting earlier steps in biofilm formation.

Discussion

The emergence of multi-drug and pan-drug resistant *A. baumannii* strains as opportunistic pathogens, in particular strains resistant to carbapenems, pose a serious threat to immunocompromised patients[24]. The need for novel chemotherapeutic interventions as treatments or prophylaxis is urgently required, and antimicrobial molecules such as glycosides with selective toxicity against antibiotic-resistant bacteria are promising candidates. HMOs and their synthetic derivatives are attractive candidates because they exhibit broad anti-adhesive properties against a variety of both gram-negative and gram-positive bacterial pathogens including *Staphylococcus aureus*, *Streptococcus agalactiae*, and *A. baumannii*[17,20,22,25,26]. Our previous study indicated that HMOs could modestly inhibit bacterial growth of a single laboratory strain of *A. baumannii* 19606T (a 6–11% reduction in growth was observed), although they were unable to inhibit biofilm formation in this strain when cultured in Todd-Hewitt broth alone or supplemented with 1% glucose. In this current work, however, we have expanded upon that study to target a wider range clinical isolates, encompassing diverse anatomical sites of isolation as well as both antibiotic susceptible and non-susceptible strains, and using a bacteriological medium (LB broth) which supports *A. baumannii* growth and biofilm better. Of the 17 isolates tested only four did not show significant reduction in biofilm formation. It is important to note that 2 of these four, isolates 16 and 112, are not strong biofilm producers. In short, HMO-dependent inhibition of biofilm is limited against strains that are low biofilm formers. In these instances, we would not expect to see reductions in biofilm formation, as little to no biofilm is formed from either of these isolates. The remaining two isolates that did not show significant reductions in biofilm formation include 13 and 32. Isolate 13 showed a large decrease in biofilm formation and while not statistically significant shows a strong trend in biofilm reduction in the presence of HMOs. Likewise, a decrease in biofilm formation was observe in isolate 32 and, again, while not statistically significant the observed decrease follows the same trend as the other tested isolates.

The utility of human milk as a prebiotic that can protect against a variety of infections has been well demonstrated in the literature[20,27,28]. Infants whose primary source of nutrition, which is derived from human breast milk, have enhanced protection against diarrhea, respiratory infection, urinary tract infection, ear infection, necrotizing enterocolitis (NEC),

and specifically, infection with *Escherichia coli*, *Clostridioides difficile*, and *Campylobacter jejuni*[29–36]. Human milk harbors a variety of antimicrobial molecules that can inhibit the growth and viability of a variety of pathogenic microbes, as well as immune-modulating molecules that influence inflammation responses to invading pathogens[34]. Our recent work and the work of others has demonstrated that the human milk glycoprotein lactoferrin has antimicrobial and antibiofilm activities against *A. baumannii* and other bacteria including *S. agalactiae*, *S. aureus*, and *Pseudomonas aeruginosa*[35–39]. In addition to the proteinaceous components of milk, the human milk glycome is also of particular interest, as components within this population of molecules have been associated with strong protection against infection by enteric pathogens[32].

Previously published data by our group demonstrates the utility of HMOs as antimicrobial and antibiofilm molecules. Additionally, we have shown that treatment with HMOs re-sensitizes the gram-positive bacterium, *S. agalactiae* to the killing action of antibiotics that it was previously found to be resistant against. Specifically, we demonstrated in *S. agalactiae*, HMOs disrupt lipid metabolism and cell membrane integrity, allowing them to penetrate into the cell[40]. Conversely, our current study reveals that HMOs do not have inhibitory effects on *A. baumannii* growth. HMOs do, however, demonstrate strong inhibition of *A. baumannii* biofilm formation (Figure 7), a result that is congruent with those observed in *S. aureus* and *S. agalactiae*[22]. It is possible that HMOs are altering metabolic pathways or target gene expression responsible for biofilm-associated virulence factors such as somatic pili, outer membrane proteins, or extracellular matrix secretion[41–44]. Interestingly, the ability of HMOs to prevent biofilm formation without initiating cell death in *A. baumannii* offers a unique opportunity to explore the anti-adhesive properties of HMOs singularly. While this work further underscores the impressive utility of these multi-faceted, naturally occurring compounds, future studies should focus on furthering our understanding of the molecular underpinnings of their pleotropic effects and broad-spectrum efficacy against bacterial biofilms on a larger, more diverse collection of isolates.

Methods

Bacterial strains and culture conditions

Eighteen strains of *A. baumannii* were utilized in this study including the laboratory reference strain 17978 (ATCC) and seventeen clinical isolates (Table 1)[2]. These strains were selected from a wide range of disease presentations including urinary tract, respiratory, wound, intra-abdominal infections, and bacteremia and diverse anatomical origin from patients. This cohort of strains represented a wide range of virulence phenotypes (including motility, biofilm formation, and hemolysis) as well as susceptibility or resistance to antibiotics. Antimicrobial susceptibility to the following antibiotics: ampicillin-sulbactam (A/S), amikacin (AK), ceftriaxone (CAX), ceftazidime (CAZ), cefotaxime (CFT), ciprofloxacin (CP), cefepime (CPE), gentamicin (GM), levofloxacin (LVX), meropenem (MER), piperacillin (PI), trimethoprim-sulfamethoxazole (T/S), tetracycline (TE), ticarcillin-K clavulanate (TIM), and tobramycin (TO) was determined as previously described[2]. Briefly, antibiotic susceptibility was determined at Nashville General Hospital using values of “susceptible”, “non-susceptible”, or “intermediate” per International Organization for

Standardization (ISO) 20776-1:2019 guidelines. A "susceptible" designation was given to bacterial strains which were inhibited *in vitro* by a clinically relevant concentration of drug. Strains exhibiting variable inhibition or inhibition by a concentration of drug associated with uncertain therapeutic effects were categorized "intermediate". Finally, "non-susceptible" strains were those in which no inhibition of growth was observed after treatment with a therapeutic concentration of drug. All isolates were grown overnight on tryptic soy agar plates supplemented with 5% sheep blood before being sub-cultured in Luria-Bertani (LB) broth (ThermoFisher) at 37 °C in room air under shaking conditions 180 rpm to an optical density of 600 nm (OD_{600}) between 0.8–1.0

Human milk oligosaccharide isolation

Expressed human breast milk was obtained from five healthy, lactating women between 3 days and 3 months postpartum and stored at −20°C. The de-identified milk samples were provided by Dr. J. Hendrik Weitkamp from the Vanderbilt University Medical Center Department of Pediatrics under a collection protocol approved by the Vanderbilt University Institutional Review Board (IRB #100897). Milk samples were centrifuged and lipid components were removed by skimming. Proteins were precipitated by addition of ethanol at 4°C and subsequent centrifugation. The HMO-containing supernatant was concentrated by vacuum dehydration, and the remaining HMO-containing extract was dissolved in 0.2 M phosphate buffer (pH 6.5) and heated to 37°C. β-galactosidase from *Kluyveromyces lactis* was added, and the reaction mixture was stirred overnight. The reaction mixture was diluted with ethanol at 4°C, centrifuged, and concentrated by vacuum dehydration. The remaining salts, glucose, and galactose molecules were separated from the oligosaccharides using size exclusion chromatography using P-2 Gel (using H_2O as the eluent), and the oligosaccharides were dried by lyophilization. Stocks of HMOs at a final concentration of 100 mg/mL were aliquoted into 1 mL tubes and frozen at −20°C until use in biological assays.

Human milk oligosaccharide characterization

MS and MS/MS analysis of HMO samples was performed as previously described. Briefly, dried HMO samples were prepared and processed for evaluation by reconstitution in water to approximately 1 mg/mL. These solutions were deposited on a matrix-assisted laser desorption/ ionization (MALDI) target plate as follows: 1 μL of HMO was spotted followed by 0.2 μL of 10 mM NaCl and 1 μL of DHB matrix (60 mg/mL in 50% methanol). The spots were allowed to air-dry and then were analyzed in positive ion mode on a 9.4T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (MS) (Bruker Solarix). Mass spectra were acquired in positive ion mode from m/z 300 to 2500. Sodium ion adducts of HMOs were detected with a mass accuracy of >2 ppm. MS/MS analysis was performed for selected ions with a linear ion trap mass spectrometer equipped with a MALDI source (LTQ XL, Thermo Scientific). Selected sodium adduct ions of interest were isolated with a 1 amu window and fragmented via CID using a collision energy of 35 eV.

Growth curve analyses

A. baumannii growth was analyzed as previously described with some modifications[2]. Briefly, overnight cultures were diluted 10-fold in fresh LB with increasing concentrations (0, 0.156 mg/mL, 0.312 mg/mL, 0.625 mg/mL, 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL) of

HMOs. Cultures were incubated at 37 °C for 24 h in shaking conditions (180 rpm). The OD_{600} was measured to quantify the density of the bacterial cells in each culture as a proxy for growth. Minimum concentration of growth inhibition (MCGI) was determined as the lowest tested concentration at which quantifiable growth inhibition was observed compared to the medium alone culture control lacking HMO supplementation, as determined by Student's *t* test comparison.

Bacterial biofilm quantification and pellicle evaluation

A. baumannii biofilms were also cultured and analyzed following a previously described protocol with some modifications[37]. Following a 10-fold dilution of overnight cultures in LB, the cultures were treated with 2.5 mg/mL of HMOs (concentrations that reflect a physiological concentration). Samples were aliquoted into the wells of a 96-well plate and incubated at 37 °C for 24 h in dark static conditions. Cultures were imaged to evaluate macroscopic pellicle formation. The OD_{600} was measured to quantify the density of the bacterial cells (biomass) in each culture. Planktonic cells were decanted and biofilms were gently washed with PBS three times before a 1% solution (w/v) of crystal violet (Sigma-Aldrich) was used to stain bacterial cells. After 30–60 minutes of staining, the solution was decanted, and washed twice with distilled water. 200 μL of 80%/20% ethanol/acetone solution (Sigma-Aldrich) was added to each well to solubilize the crystal violet and the absorbance at 560 nm was recorded. Total biofilm (as determined by OD_{560} of crystal violet stain) for each isolate was normalized to its respective total biomass by calculating the ratio of absorbance at 560 nm to 600 nm. Each biofilm assay consisted of 2–3 technical replicates and the assay was repeated at least three times using fresh overnight cultures. To determine the number of viable cells within each biofilm, quantitative culture of adherent biofilm cells was performed. *A. baumannii* strain 4 or 17978 were grown in medium alone (LB broth) or media supplemented with 5 mg/mL of HMOs for 24 hours in polystyrene tubes under static aerobic conditions. The following day, culture supernatants were decanted, biofilms were washed three times with PBS to remove planktonic cells before being scraped into fresh medium and assessed for bacterial cell viability by quantitative culture techniques to enumerate the colony forming units per mL of culture. Each assay was repeated three times with fresh biological replicates.

High resolution scanning electron microscopy assays

Bacterial biofilms were analyzed by high resolution field-emission gun scanning electron microscopy (FEG-SEM) as previously described[37]. Briefly, overnight cultures were grown in 1 mL of LB broth. The following day, fresh cultures were inoculated with a 1:100 dilution of the overnight culture in 1 mL of fresh LB broth. An abiotic substrate (polystyrene coverslips) was added to each culture upon which bacterial cells could generate a biofilm. Cultures were incubated statically at 37°C in room air to facilitate biofilm formation on the abiotic substrate for 24 hours. Planktonic cells were removed by decanting the culture and washing the biofilms gently three times with 1X PBS. Biofilms were fixed with 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.05M sodium cacodylate buffer (pH 7.4) for 24 hours before being subjected to dehydration by sequential washing with increasing concentrations of ethanol. After dehydration, samples were dried at the critical point with a Tousimis critical point dryer machine, mounted onto aluminum stubs, and then coated with

ca. 20 nm of gold by plasma sputter coating. A thin strip of colloidal silver was painted at the sample's edge to facilitate the dissipation of charge from the sample surface. Samples were imaged with an FEI Quanta 250 field-emission gun scanning electron microscope with an accelerating voltage of 5.0 KeV, a spot size of 2.5, and a working distance of 10 mm.

Evaluation of the activity of HMOs against established biofilms

A. baumannii biofilms were also cultured and analyzed following a previously described protocol with some modifications[37]. Following a 10-fold dilution of overnight cultures in LB, the cultures were aliquoted into the wells of a 96-well plate and incubated at 37 °C for 24 h in dark static conditions to allow biofilms to establish and mature. Following 24 hours post-inoculation, the media was removed from the cells and fresh LB was added with the addition of 2.5 mg/mL of HMOs (concentrations that reflect a physiological concentration). The plate was again incubated at 37 °C for 24 h in dark static conditions. Biofilm was quantified as described above.

Statistical analyses

Statistical analyses of biofilm quantifications were performed by Student's *t* test with Welch's test and one-way ANOVA with Tukey's *post hoc* test, comparing each strain's growth in medium alone versus medium supplemented with HMOs. Analyses of bacterial growth in more than two conditions were performed using one-way ANOVA with either Tukey's or Dunnett's *post hoc* test for multiple comparisons. All reported *P* values are adjusted to account for multiple comparisons. Quantitative culture results were analyzed with Student's *t* test or Mann-Whitney U test. *P* values of ≤ 0.05 were considered significant. All data analyzed in this work were derived from at least three biological replicates. Statistical analyses were performed using GraphPad Prism 6 or 8 software (GraphPad Prism Software Inc., La Jolla, California) or Microsoft Excel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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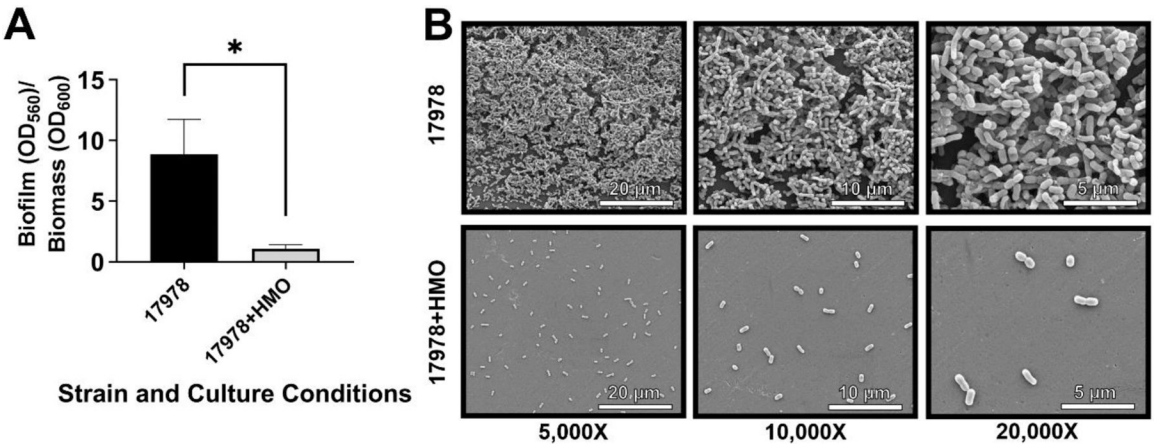

Figure 1.

Analysis of the effect of 2.5 mg/mL of human milk oligosaccharides (HMOs) on biofilm formation by *A. baumannii* 17978. Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm (OD_{600}). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm (OD_{560}) and normalizing to OD_{600} to account for cell density (Panel A). *A. baumannii* 17978 was grown in medium alone (designated by grey bars, labeled on the X-axis as 17978) or medium supplemented with 2.5 mg/mL of HMOs (designated by black bars, labeled 17978+HMO). Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by unpaired Student's *t* test, *P<0.05, n=3. Error bars represent standard error mean. (Panel B) Biofilms grown in the same conditions listed above were also analyzed by high-resolution field emission gun scanning electron microscopy (FEG-SEM) at 5,000X, 10,000X, and 20,000X magnification.

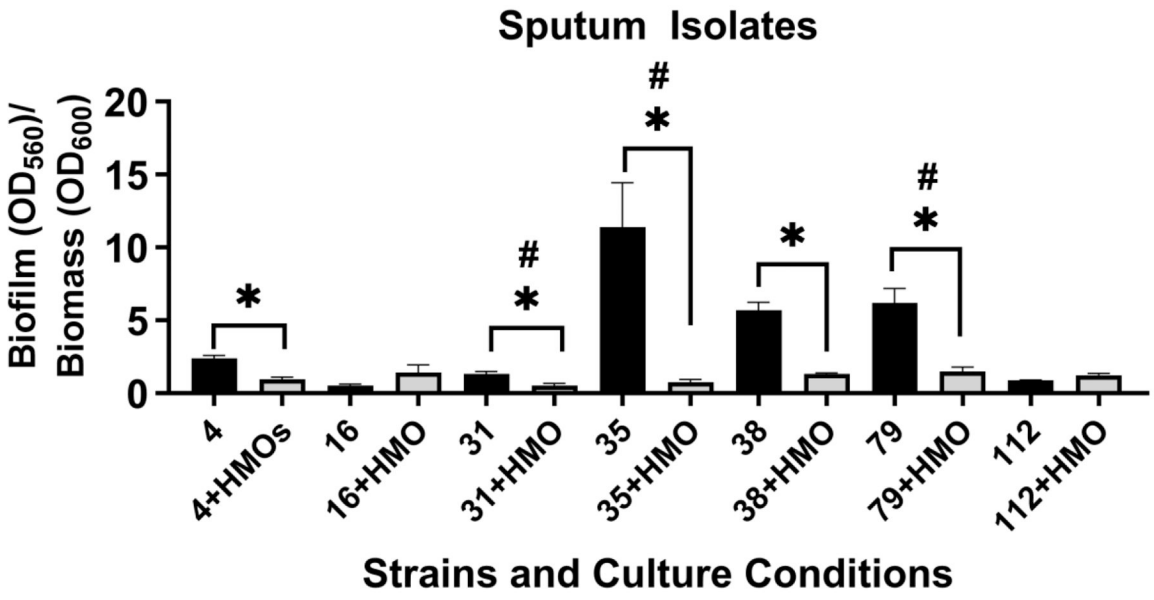

Figure 2.

Analysis of the effect of 2.5 mg/mL of human milk oligosaccharides (HMOs) on biofilm formation by clinical strains of *A. baumannii* isolated from sputum (strains 4, 16, 31, 35, 38, 79, and 112). Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm (OD_{600}). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm (OD_{560}). Cultures were grown in medium alone (designated by grey bars) or medium supplemented with 2.5 mg/mL of HMOs (designated by black bars). Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by unpaired Student's *t* test, *P<0.05, or one-way ANOVA #P<0.05, n=3. Error bars represent standard error mean.

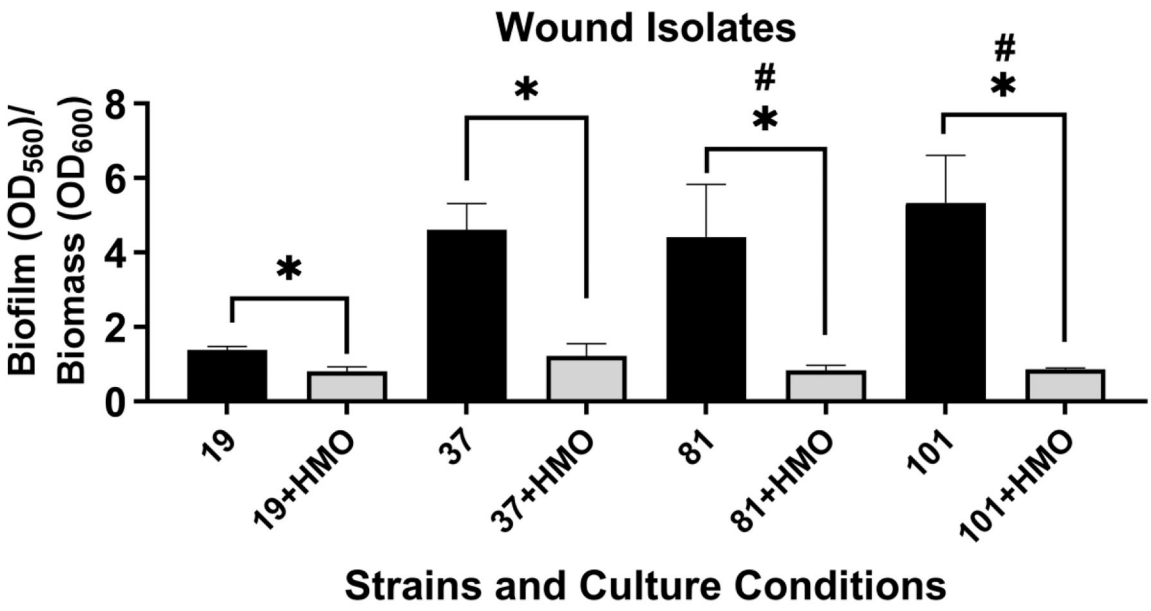

Figure 3.

Analysis of the effect of 2.5 mg/mL of human milk oligosaccharides (HMOs) on biofilm formation by clinical strains of *A. baumannii* isolated from wounds (strains 19, 37, 81, and 101). Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm (OD_{600}). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm (OD_{560}). Cultures were grown in medium alone (designated by grey bars) or medium supplemented with 2.5 mg/mL of HMOs (designated by black bars). Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by unpaired Student's *t* test, *P<0.05, or one-way ANOVA #P<0.05, n=3. Error bars represent standard error mean.

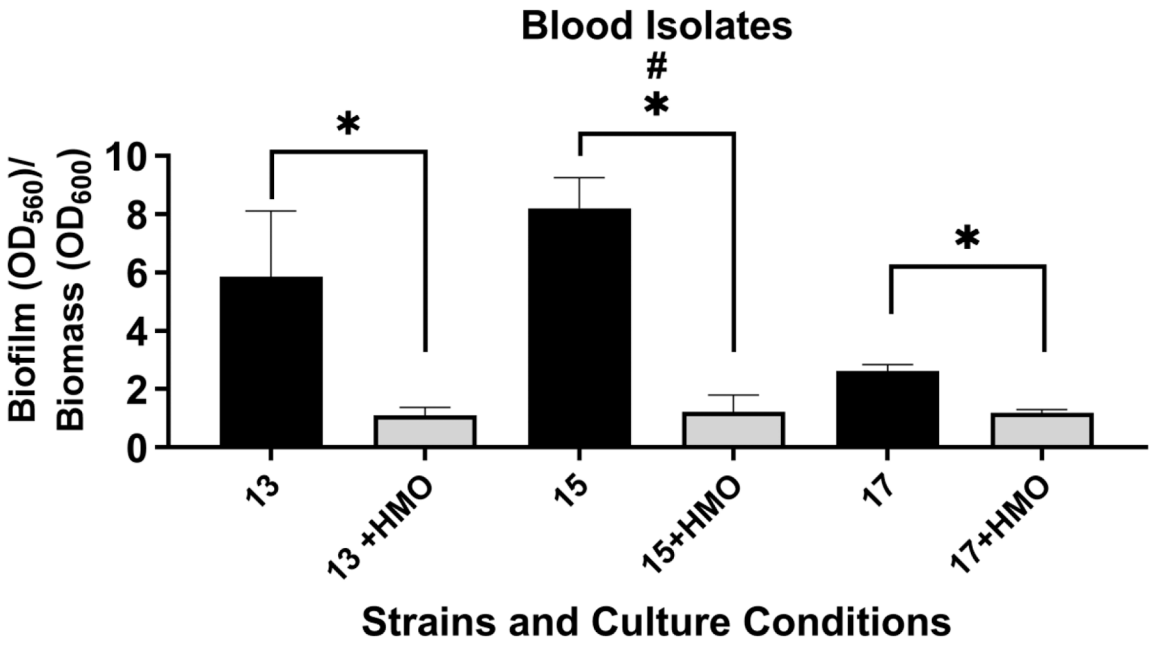

Figure 4.

Analysis of the effect of 2.5 mg/mL of human milk oligosaccharides (HMOs) on biofilm formation by clinical strains of *A. baumannii* isolated from blood (strains 13, 15, and 17). Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm (OD_{600}). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm (OD_{560}). Cultures were grown in medium alone (designated by grey bars) or medium supplemented with 2.5 mg/mL of HMOs (designated by black bars). Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by unpaired Student's *t* test, *P<0.05, or one-way ANOVA #P<0.05, n=3. Error bars represent standard error mean.

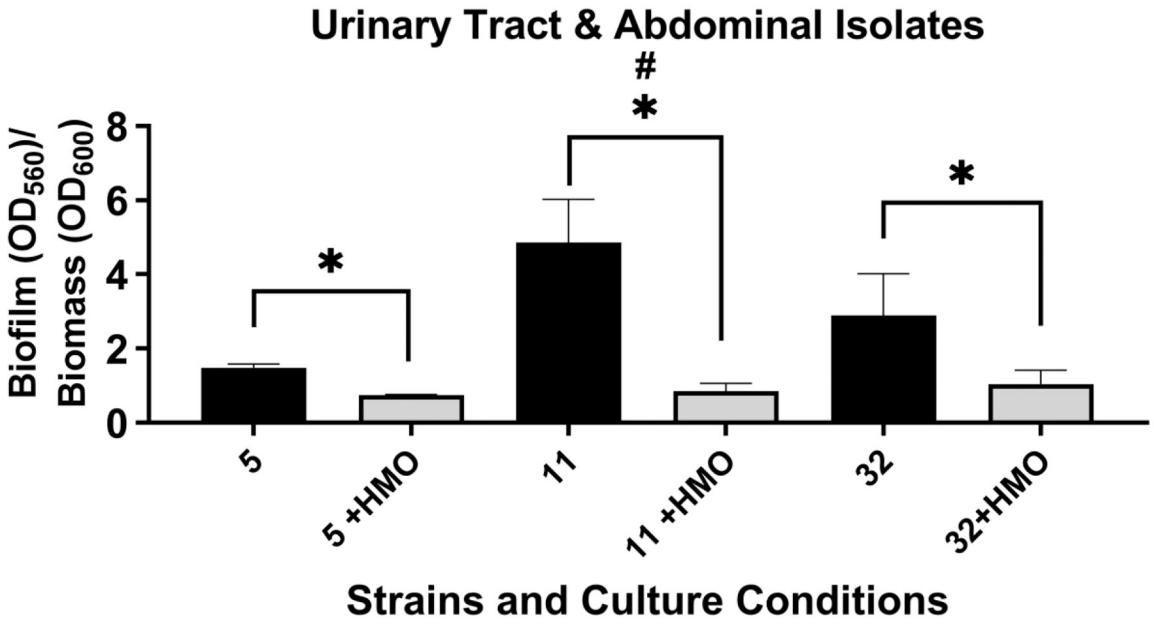

Figure 5.

Analysis of the effect of 2.5 mg/mL of human milk oligosaccharides (HMOs) on biofilm formation by clinical strains of *A. baumannii* isolated from urinary tract or abdominal cavity infections (strains 5, 11, and 32). Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm (OD_{600}). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm (OD_{560}). Cultures were grown in medium alone (designated by grey bars) or medium supplemented with 2.5 mg/mL of HMOs (designated by black bars). Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by unpaired Student's *t* test, *P<0.05, or one-way ANOVA #P<0.05, n=3. Error bars represent standard error mean.

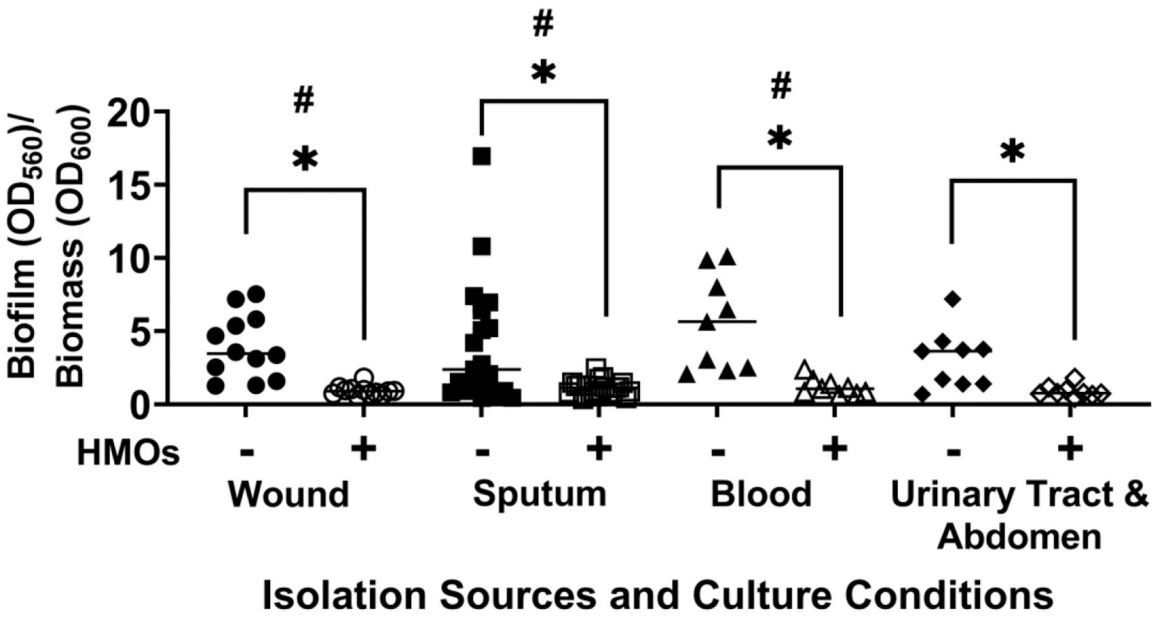

Figure 6.

Analysis of the effect of human milk oligosaccharides (HMOs) on biofilm formation by clinical strains of *A. baumannii* based on isolation source (sputum, blood, wound, urinary tract & abdominal infections). Bacterial biomass was measured at 24 hours post-inoculation by spectrophotometric measurement of optical density at 600 nm (OD_{600}). Cultures were decanted, washed, and adherent biofilms were stained with crystal violet. Biofilm was quantified by solubilizing crystal violet in an 80%/20% ethanol: acetone solution and evaluation at 560 nm (OD_{560}). Data from *A. baumannii* strains from wound (circles), sputum (squares), blood (triangles), and urinary tract or abdominal cavity infections (diamonds) were aggregated. Cultures were grown in medium alone (designated by black symbols) or medium supplemented with 2.5 mg/mL of HMOs (designated by open symbols). Significant inhibition of bacterial biofilm compared to medium alone negative control was determined by unpaired Student's *t* test, *P<0.05, or one-way ANOVA #P<0.05, n=3. Error bars represent standard error mean.

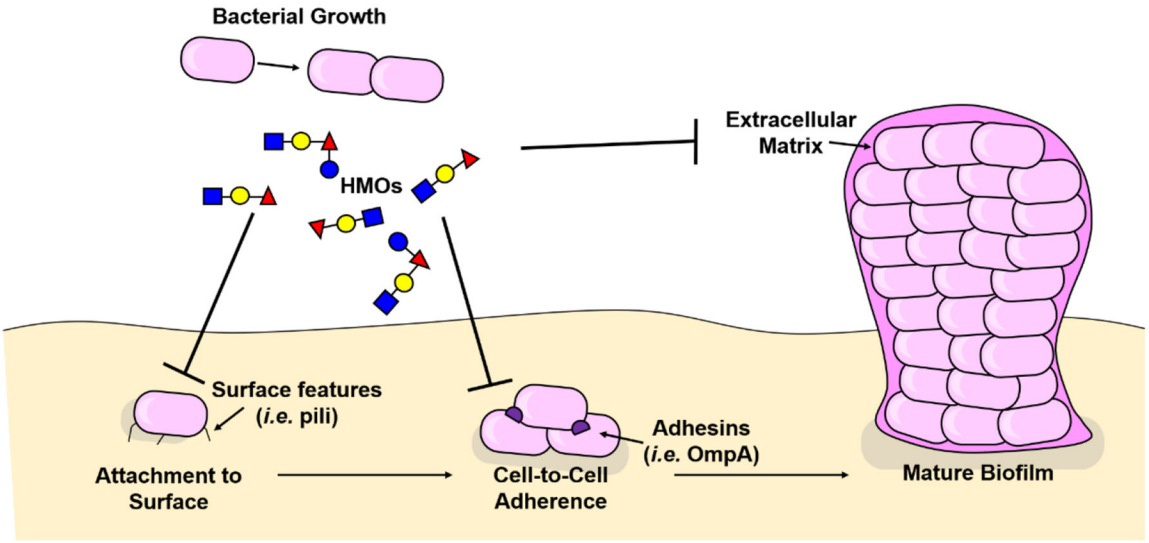

Figure 7.

Conceptual model of the anti-biofilm activity of human milk oligosaccharides (HMOs) against *A. baumannii*. HMOs do not perturb *A. baumannii* growth, but exposure to HMOs inhibits *A. baumannii* attachment to abiotic surfaces and downstream cell-to-cell adherence, and biofilm maturation. *A. baumannii* attachment to abiotic surfaces is mediated by somatic pili produced by the Csu chaperone-usher secretion system, and *A. baumannii* biotic or cellular adherence is mediated by outer membrane protein A (OmpA). Biofilm maturation includes the secretion of extracellular polymeric substances within the extracellular matrix. HMOs effectively inhibit *A. baumannii* biofilm formation.

Table 1.

A. baumannii clinical strains, isolation source, antibiotic resistance, and minimal concentration of HMOs for bacterial growth inhibition.

Isolate Number	Source	Antibiotic^{R} profile	Growth MCGI
4	Bronchial wash	A/S^{I}, AK, CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TE^{I}, TIM	>5 mg/mL
5	Abdominal Cavity	-	>5 mg/mL
11	Urine	A/S^{I}, CAX, CAZ, CFT, CP, LVX, MER^{I}, PI, T/S, TE, TIM	>5 mg/mL
13	Blood	A/S^{I}, AK, CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TE^{I}, TIM, TO	>5 mg/mL
15	Blood	A/S, AK^{I}, CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TIM, TO	>5 mg/mL
16	Sputum	CAX, CAZ, CFT, CP, CPE^{I}, GM, LVX, PI, T/S, TE^{I}, TIM^{I}, TO^{I}	>5 mg/mL
17	Blood	AK^{I}, CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TE^{I}, TIM, TO	0.625 mg/mL
19	Wound	CAX^{I}, CFT^{I}	>5 mg/mL
31	Sputum	A/S, AK, CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TE, TIM, TO	>5 mg/mL
32	Foley Catheter	A/S^{I}, CAX, CAZ, CFT, CP, CPE, GM, LVX, MER, PI, T/S, TE^{I}, TIM, TO	>5 mg/mL
35	Sputum	-	>5 mg/mL
37	Wound	A/S^{I}, AK, CAX, CAZ, CFT, CP, CPE, GM, LVX^{I}, MER, PI, T/S, TE^{I}, TIM, TO	>5 mg/mL
38	Sputum	A/S^{I}, CAX, CAZ, CFT, CP, CPE, GM, LVX^{I}, MER, PI, T/S, TIM, TO	>5 mg/mL
79	Sputum	-	>5 mg/mL
81	Wound	-	>5 mg/mL
101	Wound	-	>5 mg/mL
112	Invasive	A/S, CAX, CAZ, CFT, CP, CPE, GM, LVX, T/S, TE, TO	>5 mg/mL

Key terms and symbols. *A. baumannii* strain identification (Isolate number). Source of isolation from clinical patient (Source). Antibiotic abbreviations are as follows: Ampicillin-Sulbactam (A/S), amikacin (AK), ceftriaxone (CAX), ceftazidime (CAZ), ciprofloxacin (CP), cefepime (CPE), gentamicin (GM), levofloxacin (LVX), meropenem (MER), piperacillin (PI), trimethoprim-sulfamethoxazole (T/S), tetracycline (TE), ticarcillin-K clavulanate (TIM), and tobramycin (TO). Intermediate resistance is designated with a superscript (I). Susceptibility to all antibiotics tested is designated (-). For analyses of the minimum concentration of HMOs required to inhibit growth (MCIG), bacteria were grown in increasing concentrations of HMOs (0, 0.5, 1.25, 2.5, 5 mg/mL) and growth was measured as an optical density at 600 nm (OD_{600}) and MCIG was calculated as the lowest concentration at which quantifiable growth inhibition was observed and proven to be significant by one-way ANOVA.