Probiotic *Lactobacillus reuteri* biofilms produce antimicrobial and anti-inflammatory factors
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Abstract

**Background:** Commensal-derived probiotic bacteria inhibit enteric pathogens and regulate host immune responses in the gastrointestinal tract, but studies examining specific functions of beneficial microbes in the context of biofilms have been limited in scope.

**Results:** *Lactobacillus reuteri* formed biofilms that retained functions potentially advantageous to the host including modulation of cytokine output and the production of the antimicrobial agent, reuterin. Immunomodulatory activities of biofilms were demonstrated by the abilities of specific *L. reuteri* strains to suppress human TNF production by LPS-activated monocytoid cells. Quantification of the antimicrobial glycerol derivative, reuterin, was assessed in order to document the antipathogenic potential of probiotic biofilms. *L. reuteri* biofilms differed in the quantities of reuterin secreted in this physiological state.

**Conclusion:** *L. reuteri* biofilms secreted factors that confer specific health benefits such as immunomodulation and pathogen inhibition. Future probiotic selection strategies should consider a strain's ability to perform beneficial functions as a biofilm.

Background

Probiotics are defined by the Food and Agricultural Organization of the United Nations as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1,2]. The effectiveness of probiotics is strain-specific, and each strain may contribute to host health through different mechanisms. Probiotics can prevent or inhibit the proliferation of pathogens, suppress production of virulence factors by pathogens, or modulate the immune response. *L. reuteri* is a promising therapy for the amelioration of infantile colic, alleviation of eczema, reduction of episodes of workplace illness, and suppression of *H. pylori* infection [3-9]. *L. reuteri* is considered an indigenous organism of the human gastrointestinal tract and is present on the mucosa of the gastric corpus, gastric antrum, duodenum, and ileum [10,11].

Biofilms or adherent structured microbial communities in the oral cavity and respiratory tract are well-characterized and are associated with respiratory infections, dental caries, and periodontitis [12,13]. In contrast, biofilm-like communities of the gastrointestinal and female urogenital tracts containing beneficial lactobacilli may have a protective role. In bacterial vaginosis, indigenous lactobacilli are replaced with pathogenic biofilms consisting of *Gardnerella vaginalis* and other bacteria [6]. Probiotic *L. reuteri* can displace *G. vaginalis* biofilms and could potentially re-
establish protective biofilms in the female urogenital tract [6]. Due to artifactual removal of biofilms by traditional fixatives during specimen processing, studies of gastrointestinal biofilms are sparse. Using non-aqueous fixatives and special techniques, several groups have documented the presence of intestinal biofilms in the mammalian intestine [14-17]. The bacterial composition of biofilm-like communities in the mucus layer or the mucosa-associated microbiota (MAM), differs from the composition of the fecal microbiota, and the human MAM includes lactobacilli [18-20]. Changes in the composition and architecture of the MAM through diet or disease may affect overall health status [20-24]. Denser biofilms were found in patients with inflammatory bowel disease (IBD) when compared to healthy controls, and 60% of the biofilm mass was comprised of the commensal Bacteroides fragilis [17]. These studies indicate a need to understand the contributions of individual strains and species to the aggregate function of gastrointestinal biofilms.

This report describes the ability of an established commensal and probiotic organism, L. reuteri, to form biofilms in vitro and perform potentially beneficial functions as biofilms. Two basic probiotic functions that depend on secreted factors were studied in the context of biofilms. First, modulation of innate immunity was investigated by studying regulation of human TNF production. In prior studies, supernatants from planktonic L. reuteri cultures reduced production of the pro-inflammatory cytokine, TNF [25], and TNF suppression was important in alleviating inflammation in a murine model of IBD [26]. Probiotic L. reuteri biofilms have not been examined for TNF inhibition. Secondly, production of the antimicrobial compound β-hydroxy-propionaldehyde, known as reuterin, was evaluated in order to assess anti-pathogenic properties of L. reuteri biofilms.

Results

Probiotic Lactobacillus reuteri forms biofilms

Various human isolates of L. reuteri were grown in 96-well polystyrene plates and retention of crystal violet was used to assess relative biofilm densities (Fig. 1A). All strains of L. reuteri adhered to polystyrene, but strains differed with respect to relative densities as measured by absorbance spectrophotometry. L. reuteri strains ATCC PTA 6475 and ATCC PTA 5289 (OD<sub>570</sub> was 3.92 and 3.17, respectively) formed aggregates with greater optical densities than L. reuteri strains ATCC 55730 and CF48-3A (OD<sub>570</sub> was 1.10 and 1.44, respectively). The differences between strains were also observed in cell counts. The bacterial densities (CFU/cm<sup>2</sup>) in biofilms of ATCC PTA 6475 and ATCC PTA 5289 were roughly 10-fold greater than the bacterial densities of ATCC 55730 and CF48-3A biofilms (Fig. 1B). L. reuteri biofilms were stained with acridine orange and observed by confocal microscopy (Fig. 2). Monospecies biofilms of ATCC 55730 were 7 μm (+/- 2 μm) thick. The thickness of L. reuteri biofilms was assessed at 24 and 48 hours. No differences in biofilm thickness were observed. Consistent with this study, other researchers demonstrated formation of only thin biofilms (approximately 5 μm) when L. reuteri biofilms were cultured on plastic coupons for 32 hours [27].

L. reuteri biofilms modulate human TNF production

To test the immunomodulatory properties of L. reuteri biofilms, supernatants from the biofilms were added to human monocytic THP-1 cells in the presence and absence of LPS. LPS was added to the THP-1 cells to stimulate production of pro-inflammatory TNF by THP-1 cells. L. reuteri strains that produced TNF inhibitory factors as planktonic cultures (L. reuteri strains ATCC PTA
6475 and ATCC PTA 5289, 76 and 77%, respectively) (Fig. 3) demonstrated similar abilities to suppress TNF production when cultured as biofilms (Fig. 4). When TNF inhibitory factors were obtained directly from *L. reuteri* biofilms grown in 24-well polystyrene plates, ATCC PTA 6475 and ATCC PTA 5289 also inhibited TNF production by 60% and 50%, respectively, when compared to the media control (Fig. 4A). Supernatants of *L. reuteri* ATCC PTA 5289 biofilms cultured in a flow cell inhibited TNF by 73% compared to the media control (Fig. 4B). *L. reuteri* strains that did not suppress human TNF in planktonic phase (ATCC 55730 and CF48-3A) (Fig. 3) lacked TNF-inhibitory capabilities when supernatants were obtained from the same strains cultured as biofilms (Fig. 4). Surprisingly, supernatants from ATCC 55730 and CF48-3A biofilms did not inhibit TNF production by THP-1 cells in the absence of LPS (data not shown) as the supernatants from planktonic cultures did (Fig 3). Interestingly, the ability of probiotic *L. reuteri* to regulate human TNF production is strain-specific, and strain-specific TNF inhibition was maintained whether *L. reuteri* strains were cultured as planktonic cells or biofilms. The relative abilities to suppress human TNF in monocytoid cells were directly correlated with relative abilities to aggregate and form biofilms on polystyrene surfaces (Fig. 1A).

**Figure 2**
*L. reuteri* biofilms were observed by confocal microscopy. Biofilms were cultured in a flow cell supplied with MRS for 48 hours at 37°C in ambient atmosphere. *L. reuteri* biofilms (green) were stained with acridine orange and observed by confocal microscopy. A single optical section and the stacked optical sections of ATCC 55730 (A and B, respectively) are shown at 630× magnification. These images are representative of 30 microscopic fields obtained in 3 independent experiments.

**Figure 3**
Modulation of TNF production by *L. reuteri* is strain-dependent. Cell-free supernatants from stationary phase *L. reuteri* cultures (planktonic cells) were added to human monocytoid cells in the presence or absence of *E. coli*-derived LPS (no LPS-black bars, LPS-gray bars). Quantitative ELISAs measured the amounts of human TNF produced by THP-1 cells. In the absence of LPS, supernatants from *L. reuteri* CF48-3A and ATCC 55730 stimulated TNF by human THP-1 cells, while supernatants from ATCC PTA 6475 and ATCC PTA 5289 did not induce TNF production. However, *L. reuteri* CF48-3A and ATCC 55730 did not suppress TNF production by LPS-activated cells, while PTA 6475 and ATCC PTA 5289 inhibited production of TNF by 76% and 77% respectively, when compared to the media control (ANOVA, p < 0.001).

**Figure 4**
Antimicrobial activities of *L. reuteri* were assessed by examining supernatants of planktonic and biofilm cultures for reuterin. Planktonic cells and biofilms of *L. reuteri* produced reuterin, although differences in reuterin production were evident among strains. Planktonic cultures of ATCC PTA 6475, ATCC PTA 5289, ATTC 55730 and CF48-3A produced 51.2, 45.2, 225.9, and 230.3 mM reuterin.
of reuterin, respectively. When reuterin quantities were normalized to initial CFU/mL, planktonic cultures of ATCC PTA 6475 and ATCC PTA 5289 produced 2.32 and 2.3 mmol reuterin/10^{12} cells, respectively, and ATCC 55730 and CF48-3A produced 31.89 and 36.24 mmol reuterin/10^{12} cells, respectively (Fig. 5). For biofilms cultured in multiwell plates, the four wild type L. reuteri strains ATCC PTA 6475, ATCC PTA 5289, ATCC 55730 and CF48-3A produced 26.8, 16.5, 19.1, and 22.1 mM of reuterin, respectively. After normalization of reuterin quantities to bacterial cell counts, ATCC PTA 6475, ATCC PTA 5289, CF48-3A, and ATCC 55730 produced 6.61, 5.41, 43.4, and 53.94 mmol of reuterin/10^{12} cells, respectively, when cultured as biofilms in multiwell plates (Fig. 6). Trends in reuterin production were consistent with planktonic and biofilm cultures of ATCC PTA 6475 and ATCC 5289 producing lower quantities of reuterin than strains ATCC 55730 and CF48-3A. Interestingly, the relative abilities of L. reuteri strains to produce reuterin were inversely correlated with relative abilities to aggregate and adhere to polystyrene (Fig. 1A).

Previous studies indicated that planktonic cultures of human-derived L. reuteri strains used in this study were relatively resistant to the antimicrobial effects of reuterin (10 mM), when compared to other bacterial species including closely related lactobacilli [29,43] and JK Spin-
innate immune system may be associated with enhanced protection against enteric infections.

Interestingly, mucosal inflammation has been associated with enhanced biofilm densities in the intestine [34,35]. The pro-inflammatory cytokine TNF promotes the proliferation of E. coli, and secretory IgA increased agglutination of E. coli, an initial step in biofilm development [34,36,37]. Although, these experiments were performed with monospecies biofilms in vitro, the data raise questions regarding events that occur in complex microbial communities in vivo. When not attached to a surface, immunostimulatory L. reuteri strains may stimulate host immune responses and promote commensal biofilm formation, particularly in neonates. When L. reuteri biofilms are established, probiotic strains may have a diminished ability to stimulate TNF, effectively suppressing the formation of dense, complex multispecies biofilms in the mucus layer. Because such complex, dense biofilms have been associated with inflammation and disease [17], the ability of probiotics to differentially regulate production of immunomodulatory factors in the context of planktonic and biofilm lifestyles may be an important probiotic feature. Alternatively, the TNF stimulatory factor(s) may be produced by L. reuteri biofilms and not detected in the experimental conditions used in this study. In contrast to immunostimulatory L. reuteri strains, anti-inflammatory probiotics may form denser biofilms in vivo that thwart pathogenic biofilm formation by preventing harmful host:pathogen interactions and overgrowth of commensal bacteria in the intestine. As an example of pathogen inhibition, other lactobacilli suppressed the binding of Staphylococcus aureus to epithelial cells [38].

Reuterin is a potent anti-pathogenic compound produced by L. reuteri and capable of inhibiting a wide spectrum of microorganisms including gram-positive bacteria, gram-negative bacteria, fungi, and protozoa [39]. Maximum reuterin production by L. reuteri occurs during late log and stationary phase cultures (J.K. Spinler, personal communication). Reuterin and other anti-pathogenic factors may be important for maintaining a healthy gut microbiota by preventing intestinal overgrowth by other commensal and pathogenic microorganisms. Recently, the addition of L. reuteri ATCC 55730 or reuterin to the intestinal microbiota was shown to reduce the E. coli population in an in vitro fermentation model [40]. Thus, antimicrobial compounds like reuterin may have a fundamental role in shaping and modeling the composition and spatial architecture of the gastrointestinal microbiota. L. reuteri biofilms produced reuterin, indicating that probiotic L. reuteri may be protective against pathogens in either the planktonic or biofilm state. Interestingly, strains that produce relatively high quantities of reuterin are immunostimulatory when cultured as planktonic cells. In vivo,
intestinal microbiota may alter intestinal physiology and community architecture, or composition of the gastrointestinal microbial factors. Changes in the spatial distribution, that is shaped and maintained by multiple host and human gastrointestinal microbiota is a complex ecosystem that is shaped and maintained by multiple host and microbial factors. Changes in the spatial distribution, community architecture, or composition of the gastrointestinal microbiota may alter intestinal physiology and immunostimulation by *L. reuteri* may promote colonization and biofilm formation of commensal lactobacilli, and reuterin could prevent opportunistic bacteria from establishing a niche. Hypothetically, once the immunostimulatory strains are established on the mucosal surface, TNF stimulation is diminished, and higher quantities of reuterin are produced. Elevated quantities of reuterin adjacent to the mucosa may effectively alter surrounding commensal microbial populations and prevent colonization and adherence by pathogenic bacteria. Biofilms are relatively resistant to several antimicrobial agents when compared to planktonic cultures [41]. The enhanced resistance of biofilms to antimicrobial compounds may explain, in part, the resistance of *L. reuteri* biofilms to reuterin and elevated amounts of reuterin produced by these biofilms, as described in this study.

While the growth conditions used for the flow cell and planktonic cultures differed, similar probiotic activities by each *L. reuteri* strain were observed. TNF inhibitory activities and reuterin production of *L. reuteri* were also consistent when biofilms (in multiwell plates) and planktonic cells were cultured using the same growth conditions. Although these experiments were conducted with biofilms grown in vitro on abiotic surfaces, biofilms with probiotic function may be important for delivery of beneficial effects in the mammalian host. A mutant strain of *L. crispatus*, unable to bind mucus and adhere to the colonic mucosa, did not have a protective effect in a murine colitis model compared to the wild type aggregating strain even when the bacteria were continuously supplied to mice [42]. Mucus-binding ability may be important for probiotics to adhere to the mucosal surface and form biofilms within the intestine. Defects in cell surface features may affect biofilm formation and the abilities of probiotics to persist and colonize the intestine in vivo. *L. reuteri* strain 100–23 deficient in alanylation of cell surface lipoteichoic acids proliferated similarly to wild type cells as planktonic bacteria, but failed to compete effectively and form complex biofilms in vivo [43]. In the context of established intestinal microbial communities, probiotic biofilms may be more effective at long-term colonization and restoring missing functions in disease states.

**Conclusion**

In conclusion, probiotic strategies for the prevention and treatment of disease may require discovery and development of strains that form effective biofilms. If biofilm formation facilitates long-term colonization and persistence in the intestine, biofilms that retain probiotic functions may be important for sustained efficacy in vivo. The human gastrointestinal microbiota is a complex ecosystem that is shaped and maintained by multiple host and microbial factors. Changes in the spatial distribution, community architecture, or composition of the gastrointestinal microbiota may alter intestinal physiology and immunity, including susceptibility to infection. Probiotics in biofilm-like communities may be essential for long-term remodeling of the composition and function of the intestinal microbiome.

**Methods**

**Key reagents, bacterial strains and mammalian cell lines**

*L. reuteri* strains were grown in deMan, Rogosa, Sharpe (MRS; Difco, Franklin Lakes, NJ) or LDMIIIG (pH 6.5) (see Additional file 1) media. An anaerobic chamber (1025 Anaerobic System, Forma Scientific, Waltham, MA) supplied with a mixture of 10% CO₂, 10% H₂, and 80% N₂ was used for anaerobic culturing of lactobacilli. Biogaia AB (Raleigh, NC) provided *L. reuteri* strains ATCC PTA 6475, ATCC PTA 5289, ATCC 55730, and CF48-3A. *L. reuteri* ATCC PTA 6475 and ATCC 55730 were isolated from the breast milk of healthy Finnish and Peruvian women, respectively. ATCC PTA 5289 is an oral isolate from a healthy Japanese woman. CF48-3A was isolated from the feces of a healthy Finnish child. THP-1 cells (ATCC TIB-202) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37 °C and 5% CO₂. All chemical reagents were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Polystyrene 96- and 24-well plates for biofilm and tissue culture studies were obtained from Corning (Corning, NY). Filters with polyvinylidene fluoride membranes (0.22 mm pore size) (Millipore, Bedford, MA) were used for sterilization.

**L. reuteri biofilm adherence studies**

*L. reuteri* cultured in MRS media for 16–18 hours were diluted 1:50 in MRS to a final volume of 200 μL in sterile 96-well polystyrene plates. Plates were incubated anaerobically at 35 °C for 24 hours. Media and planktonic cells were removed by aspiration and two washes with de-ionized water. Adherent cells were stained with crystal violet (0.1% w/v) for 15 minutes at 37 °C, 200 rpm. Crystal violet was discarded and the plates were washed with de-ionized water. The crystal violet was redissolved with ethanol and the OD₅₇₀ was determined by absorbance spectrophotometry using a Spectramax 340 PC [384] (Molecular Devices, Sunnyvale, CA).

**Confocal imaging of L. reuteri biofilms**

Glass flow cells with a volume of 7.7 mL (Stovall Inc., Greensboro, NC) were assembled according to the manufacturer’s instructions and maintained at 37 °C in ambient atmosphere. As previously described, one mL of *L. reuteri* (OD₆₀₀ = 0.1 or 7 × 10⁷ cells) was injected into the flow cell [44]. *L. reuteri* were allowed to adhere to the glass surface for an hour before being continuously supplied with 25% MRS (v/v) at 2 mL per minute. Cell counts verified that the selected flow rate removed planktonic cells and retained adherent bacteria on the surface of the flow cell. After 48 hours, the flow cells were collected and washed
once with sodium phosphate buffer (50 mM) for 10 minutes at 37°C, 70 rpm. *L. reuteri* biofilms were stained with acridine orange for imaging by confocal microscopy.

**Preparation of cell-free supernatants from *L. reuteri* planktonic cultures for immunomodulation studies**

For planktonic cells, 10 mL of LDMIIIG was inoculated with *L. reuteri* cultures (incubated 16–18 hrs) and adjusted to OD<sub>600</sub> = 0.1. Bacteria were incubated for 24 hours at 35°C in anaerobic conditions. Cells were pelleted (4000 × g, RT, 10 minutes) and discarded. Supernatants were filter-sterilized (0.22 μm pore size) and stored at 4°C before the concentration of reuterin was determined. The quantities of reuterin were determined using a colorimetric assay previously described [46]. Briefly, serial dilutions of reuterin were made in sterile glycerol (250 mM). Forty μL of each reuterin dilution were combined with 30 μL of tryptophan (10 mM) in HCl (50 mM), and 120 μL of HCl (12 M). Under acidic conditions, tryptophan reacts with the aldehyde of reuterin to form a β-carboline derivative that oxidizes to yield a purple pigment. Plates were incubated for 25 minutes at 37°C in ambient atmosphere, and the OD<sub>560</sub> was determined using the Spectramax 340 PC384 (Molecular Devices, Sunnyvale, CA). Dilutions of HPLC-quantified reuterin were used as standards. The amount of reuterin produced was normalized to the initial CFU/mL of the cultures.

**Preparation of cell-free supernatants from *L. reuteri* biofilms for immunomodulation studies**

For biofilms grown in 24-well plates, *L. reuteri* cultures (16–18 hrs of incubation) were diluted 1:100 in 1 mL of MRS broth. Plates were incubated anaerobically for 24 hours at 35°C. Supernatants and planktonic cells were removed by aspiration, and biofilms were washed with 50 mM sodium phosphate buffer (37°C, 100 rpm, 10 minutes). One mL of LDMIIIG was added to each well, and the plates were incubated for 2 hours at 35°C in anaerobic conditions. The supernatants were filter-sterilized (0.22 μm pore size), vacuum-dried and resuspended in RPMI to the starting volume.

*L. reuteri* biofilms were cultured in flow cells supplied with MRS media for the first 23 hours followed by immersion in LDMIIIG at a flow rate of 2 mL per min in ambient atmosphere at 37°C. Biofilm supernatants were collected by sampling effluents, downstream from the chambers containing the biofilms, at the flow cell’s luer lock connection after 24 hours of culture. The supernatants were filter-sterilized (0.22 μm pore size), vacuum dried, resuspended to 1/20 the starting volume in RPMI, and tested for TNF inhibition.

**TNF inhibition experiments**

As previously described [45], cell-free supernatants of *L. reuteri* planktonic cell or biofilm cultures (5% v/v) and *E. coli* O127:B8 LPS (100 ng/mL) were added to human THP-1 cells (approximately 5 × 10<sup>4</sup> cells). Plates were incubated at 37°C and 5% CO<sub>2</sub> for 3.5 hours. THP-1 cells were pelleted (1500 × g, 5 minutes, 4°C), and TNF quantities in monocytoid cell supernatants were determined by quantitative ELISAs (R&D Systems, Minneapolis, MN).

**Preparation of cell-free supernatants from *L. reuteri* planktonic cultures for reuterin quantification**

Based on a previously described method [46], *L. reuteri* cultures (16–18 hrs of incubation) were washed twice with sodium phosphate buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub>). The cells were then suspended in sterile 250 mM glycerol at 2.2 × 10<sup>9</sup> CFU/mL and incubated anaerobically at 35°C for 2 hours. Supernatants were filter-sterilized (0.22 μm pore size) and stored at 4°C.

The concentration of reuterin was determined. Reuterin produced was normalized to the initial CFU/mL of the cultures.
Additional material

**Additional file 1**

Supplementary table. The recipe for the medium, LDML111G. Click here for file

[http://www.biomedcentral.com/content/supplementary/1471-2180-9-35-S1.doc](http://www.biomedcentral.com/content/supplementary/1471-2180-9-35-S1.doc)

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