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Potentiated In Vitro Probiotic Activities of *Lactobacillus fermentum LfQi6* Biofilm Biomass Versus Planktonic Culture

Eva Berkes[1,2] · Yu-Hsien Liao[1] · Daniel Neef[1] · Michael Grandalski[1] · Nicholas Monsul[1,2]

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Abstract
In this study, we describe enhanced in vitro probiotic activities of preformed biofilms versus planktonic cultures of *Lactobacillus fermentum LfQi6* (*LfQi6*), a lactic acid bacterium (LAB) isolated from the human microbiome. These evaluations are used to help predict host in vivo probiotic benefits and therefore indicate that *LfQi6* may provide significant probiotic benefits in the human host when administered as preformed biofilms rather than as planktonic cultures. Specifically, *LfQi6* biofilms demonstrated improved in vitro performance versus *LfQi6* planktonic cultures for host gastrointestinal survival and engraftment, strain-specific antimicrobial and anti-biofilm activity against clinically significant pathogens, concurrent promotion of beneficial gastrointestinal commensal biofilms, beneficial commensal enzyme activities, and host cellular-protective glutathione antioxidant activity. Evaluation of *LfQi6* according to the European Food Safety Authority (EFSA 2007, 2012, 2015) Guidelines and Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Evaluation of Probiotics in Food (FAO/WHO, 2002) demonstrates strain safety. In summary, in vitro evaluation of *Lact. fermentum LfQi6* demonstrates significant evidence for strain-specific probiotic characteristics and safety. Moreover, strain-specific as well as biofilm-phenotype-specific benefits demonstrated in vitro furthermore suggest that in vivo use of *LfQi6* biofilm biomass may be of greater benefit to the human host than the use of standard planktonic cultures. This concept – potentiating probiotic benefits through the use of preformed commensal biofilms – is novel and may serve to further broaden the application of microbial biofilms to human health.

Keywords Probiotic · Biofilm · Lactobacillus · Microbiome · Microbiota · Pathogen

Introduction

The World Health Organization (WHO) has defined probiotics as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" [1]. The human body has evolved necessarily redundant physical, chemical, and cellular antimicrobial defense systems, including extensive colonization of body surfaces by microbiota, such as the bacterial symbionts *Lactobacillae*, also known as lactic acid bacteria (LAB). In exchange for host nutrients and a protected growth environment on human body surfaces, numerous LAB-associated benefits have been observed: inhibition of pathogen colonization, organ barrier integrity maintenance, and modulation of inflammation [2, 3]. Extensive work has repeatedly shown that endowment of probiotic benefits is strain-specific. While the strain-dependent effect of probiotic organisms is well-studied, much less is known about how other microbial phenotypic variables might affect probiotic characteristics. Such variables include culture conditions such as media composition, temperature and oxygenation, and planktonic versus biofilm microbial growth modes. A biofilm is a structured microbial community adhered to an inert or living surface and embedded in a self-produced extracellular polymeric substance [4]. This phenotype contrasts against that of planktonic microbes, which function as single units, traditionally evaluated via standard agar plating and liquid culture [5]. Biofilm has different gene expression and protein production than when the same organism is grown planktonically [6]. While to date, disease-associated biofilms remain the most studied, such growth mode functional differences are also likely to be seen in commensal biofilms. The hypothesis

✉ Eva Berkes
evaberkesmd@quoruminnovations.com

[1] Quorum Innovations, LLC, 2068 Hawthorne Street, Sarasota, FL 34239, USA

[2] Florida State University College of Medicine Clerkship Faculty Sarasota Regional Campus, Sarasota, FL, USA

evaluated in this communication is that probiotic microbiota cultured as biofilms may demonstrate unique and/or potentiated human health benefits versus same-strain planktonic cultures. This hypothesis is tested using a proprietary human commensal *Lact. fermentum* strain *LfQi6* (*LfQi6*), previously identified by our lab, with accession number *LAIK00000000.1* [1]. Combining in vitro biofilm techniques with standard in vitro methodology used to assess probiotic efficacy and safety, we compare and contrast the probiotic properties of preformed *LfQi6* biofilm against planktonic biomass.

Materials and Methods

Bacterial Strains, Human Cell Lines, and Culture Conditions

LfQi6 was isolated from the human microbiome in a previous study [7]. *Lactobacillus rhamnosus GG* was isolated from Culturelle Probiotics®. Both lactobacilli were routinely cultured in deMan, Rogosa, and Sharp (MRS) broth (Sigma, MO) at 37 °C. *LfQi6* was grown as a biofilm using a solid support for biofilm establishment. *E. coli* (ATCC®, 11,775), MRSA (ATCC® BAA-44), *Staphylococcus aureus* (ATCC®, 33,591), *Pseudomonas aeruginosa* (ATCC® 10,145), and *Klebsiella pneumonia* (in-house clinical isolate) were grown in Luria-Bertani (LB) and tryptic soy broth (TSB) (Sigma, MO), respectively. *S. enterica subsp. enterica* (*Kauffman and Edwards*) *Le Minor and Popoff (*ATCC 51741) was grown in brain-heart infused media. Unless otherwise specified, planktonic cultures were obtained at mid-log phase. Caco-2 cells were obtained from ATCC® (HTB-37) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and antibiotic/anti-mycotic solution (Sigma, MO). Unless otherwise indicated, all experimental reagents were purchased from Sigma, MO.

Phylogenetic Analyses

Phylogenetic multiple sequence alignments were performed using the 16S rRNA sequences of the indicated *Lactobacillus* strains on Clustal Omega [8, 9]. For *Lact. fermentum* isolate alignments, whole-genome alignments were performed using the NCBI whole-genome alignment tool [10].

Acid Resistance

Both planktonic and biofilm cultures were harvested, washed, and resuspended in phosphate buffered saline (PBS) at pH 7.2, pH 3.0, or pH 2.0 and incubated for 3 h at 37 °C. Aliquots (100 μl) were removed at time points 0 and 3 h and plated on MRS agar for cell viability counting via standard colony-forming units per milliliter (CFU/ml) enumeration. All counts were obtained in duplicate and expressed as mean ± SD.

Bile Salt Tolerance

MRS broth supplemented with 0.2% thioglycolate and increasing concentrations of Oxgall bile salts was inoculated with *LfQi6* at 1×10^6 CFU/ml and grown overnight at 37 °C. The following day, aliquots (100 μl) were plated on MRS agar for colony-forming unit (CFU) counting. All counts were obtained in duplicate and expressed as mean ± SD.

Bile Salt Hydrolase (BSH) Activity

Bacteria (10 μl) from an overnight planktonic or biofilm culture were spotted on MRS agar supplemented with 0.5% taurodeoxycholic acid (TDCA) and 0.37 g/L $CaCl_2$ and incubated anaerobically at 37 °C for 48 h. All counts were obtained in duplicate and expressed as mean ± SD. BSH activity is evidenced by the formation of a white precipitate on or around the bacterial colony.

Survival in Simulated Fasted and Fed Human Intestinal Media

To help predict planktonic versus biofilm survival in the fasted versus fed human intestinal tract, the most widely used biorelevant media modeling in vivo intestinal lumenal conditions was used [11]. A commercial assay and accompanying protocol were followed (Biorelevant LTD, London, England). Briefly, *Lact. fermentum* Qi6 was grown in biofilm or planktonic phenotype (8 h to mid-log phase), and 10^8 CFU were pelleted, washed in PBS, and resuspended in 1 ml of either PBS, FaSSIF (fasting state intestinal fluid: bile salts, lecithin, sodium hydroxide, sodium phosphate, sodium chloride, pH 6.5), FeSSIF (fed state intestinal fluid: bile salts, lecithin, sodium hydroxide, acetic acid, sodium chloride, pH 5), or FaSSGF (fasting state gastric fluid: bile salts, lecithin, sodium chloride, pH 1.6). After 2-h incubation at 37°, bacteria were diluted in PBS and plated on MRS agar for CFU counting. All counts were obtained in duplicate and expressed as mean ± SD.

Cholesterol Measurement

Water-soluble cholesterol-PEG600 was added to MRS broth to a final concentration of 50 μg/ml, supplemented with 0.2% thioglycolate and 0.3% Oxgall, and inoculated (1% *v*/*v*) with either an overnight culture of *LfQi6* or MRS broth without bacteria. Cultures were incubated for 18 h at 37 °C, the bacteria were removed by centrifugation (4500 rpm; 10 min), and

cholesterol concentration in the growth media was measured using the EnzyChrom Cholesterol Assay Kit (BioAssay Systems, CA), according to the manufacturer's instructions. All counts were obtained in duplicate and expressed as mean ± SD.

Cell Surface Hydrophobicity (CSH) Determination

A bacterial adhesion to hydrocarbon test (BATH) was performed to assess CSH [12]. Briefly, overnight cultures were washed and resuspended in 3-ml PBS, pH 7.4 to an OD_{600} = 0.4 (A_0). Xylene (1 ml) was added to the cell suspension and incubated for 10 min, after which, the two phase systems were mixed by vortexing for 2 min. The two phases were allowed to separate for 20 min at 37 °C at which time the OD_{600} of the aqueous phase was measured (A_1). All counts were obtained in duplicate and expressed as mean ± SD. Percent hydrophobicity was calculated as follows:

$$\%Hyd = (1–A_1/A_0)\, x\, 100$$

Auto-Aggregation and Co-aggregation Assays

To measure auto-aggregation, overnight *LfQi6* cultures were washed and resuspended in PBS to an OD_{600} = 0.4 (A_0) and incubated without shaking at 25 °C. The OD_{600} was measured after 3, 5, and 24 h (A_t). All counts were obtained in duplicate and expressed as mean ± SD. Percent auto-aggregation was calculated as follows:

$$\%AutoAgg = (A_0–A_t)/A_0 x\, 100$$

To measure co-aggregation, overnight cultures of *LfQi6*, *E. coli*, and MRSA were washed and resuspended in PBS to an OD_{600} = 0.4. Next, 2.5 ml of the *LfQi6* suspension was mixed with 2.5 ml of either the *E. coli* or MRSA cultures. Akin to the auto-aggregation studies, co-culture suspensions were incubated at 25 °C without shaking, and OD_{600} was measured at 3, 5, and 24 h for the individual suspensions as well as the mixed suspensions. All counts were obtained in duplicate and expressed as mean ± SD. For each time point, the percent co-aggregation is determined as follows:

$$\%CoAgg = (Co_0–Co_t)/Co_0 x\, 100$$

The predicted absorbance of the mixed suspension (A_P) is determined by calculating the average of the *LfQi6* suspension absorbance (A_{LfQi6}) and the pathogen suspension absorbance (A_{path}) as follows:

$$A_P = \left(A_{LfQi6} + A_{path}\right)/2$$

The actual observed absorbance of the mixed suspension is then measured as A_O. All counts were obtained in duplicate and expressed as mean ± SD. Percent co-aggregation is calculated by the deviation of the predicted absorbance from the observed absorbance as follows:

$$\%CoAgg = \left(A_P–A_{O}\right)/A_O x\, 100$$

Human Intestinal Cell Monolayer (Caco-2) Binding Assays

Caco-2 cells were seeded at 5×10^5 cells per well and maintained for 2 weeks to allow for differentiation into a monolayer of polarized intestinal epithelial cells prior to use in cell adhesion assays. Binding of *LfQi6* to Caco-2 cells was assayed by adding 10^8 CFU/ml of planktonic or biofilm-derived *LfQi6* cultures to the wells containing the Caco-2 monolayer. After 3-h incubation at 37 °C, 5% CO_2, Caco-2 cells were washed 3 times in PBS and lysed with 1% Triton X-100 to release bound bacteria. The binding ratio was calculated by plating the released bacteria on MRS agar for CFU counting and determining the number of *LfQi6* CFU bound/Caco-2 cells. For microscopic analyses (100X magnification; Olympus BX60), Caco-2 cells as well as the adhered bacteria were fixed in methanol and stained with 0.1% crystal violet. *LGG* was used as positive control for Caco-2 binding. All counts were obtained in triplicate and expressed as mean ± SD.

Feruloyl Esterase Activity (FEA) Assay

Bacteria were grown overnight in MRS broth supplemented with 1.33-mM ethyl ferulate, and 10 μl was spotted on MRS agar supplemented with 0.15% ethyl ferulate. Feruloyl esterase activity (FEA) is evident as a halo around the colony. To assess protease activity, 10 μl of an overnight culture was spotted on MRS agar supplemented with 1% milk casein. All counts were obtained in duplicate and expressed as mean ± SD.

Cell Free Supernatant (CFS) Antimicrobial Activity

Cell free supernatant (CFS) was generated by growing *LfQi6* for 48 h in MRS broth. Bacterial cells were removed by centrifugation and passed over a 0.2-μM filter (0.22-μM pore size SLGV R25 KS filter, EMD Millipore Corp, USA). Methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa* were aliquoted into a 96-well plate (100 μl; 1×10^5 CFU/ml) and treated with either 100-μl PBS or 100-μl *LfQi6* CFS and incubated overnight at 37 °C. The following day, 100 μl

aliquots were removed and plated on MRS agar for CFU counting. All counts were obtained in duplicate and expressed as mean ± SD.

LfQi6 Antimicrobial Activity

The indicated pathogens and *LfQi6* were cultured overnight in LB and MRS media, respectively. MRS soft top agar (0.75 g/L) was prepared and allowed to cool to 50 °C at which point 1 ml of pathogen overnight culture was added to 50 ml MRS soft top agar. Aliquots (10 mls) of the pathogen-inoculated soft top agar were dispensed onto the surface of an MRS plate and allowed to solidify. *LfQi6* planktonic cell overnight culture was spotted (10 μl) onto each pathogen plate and incubated overnight at 37 °C. Zones of inhibition were measured from the edge of *LfQi6* colony to the edge of the zone of inhibition. All tests were performed in triplicate and results expressed as mean ± SD.

Glutathione Colorimetric Microplate Assay

LfQi6 and LGG were cultured overnight and harvested the same cell mass of planktonic and biofilm cultures used for protein extraction (Lowry protein assay; Thermo Fisher Scientific). Supernatants containing 4 μg/μL of protein were used for glutathione microplate assay, and the assay performed according to the manufacturer's instructions (Invitrogen Colorimetric Detection Kit, Cat. No. EIAGSHC, Thermo Fisher Scientific). The same kit was used for all assays. Samples were performed in triplicate and results expressed as mean ± SD.

Safety Assessments

Antibiotic Susceptibility Studies

Antibiotic susceptibility was investigated using BD BBL™ Sensi-Disc™ antimicrobial susceptibility test discs, according to the manufacturer's instructions. Briefly, OD_{600} values were compared with the control using antibiotic-free MRS broth in a twofold broth microdilution method [9]. The antibiotics tested were ampicillin [10 μg], amoxicillin with clavulanic acid (Amoxi-Clav; 20/10 μg), cefoxitin [30 μg], chloramphenicol [30 μg], ciprofloxacin [5 μg], clindamycin [2 μg], daptomycin [30 μg], erythromycin [15 μg], fosfomycin [200 μg], gentamycin [10 μg], imipenem [10 μg], linezolid [30 μg], meropenem [10 μg], oxacillin [1 μg], penicillin G [10 U], rifampin [5 μg], tetracycline [30 μg], SMZ-TMP [5 μg], and vancomycin [30 μg], in accord with EFSA 2012 recommendations [13, 14]. Overnight cultures were resuspended in MRS broth at an approximately OD_{600} of 1. A volume of the obtained suspension (100 μl) was inoculated into MRS broth containing the selected antibiotic and incubated at 30 °C. Plates were examined after 18 h of incubation, per test instructions, and zones of inhibition were recorded to the nearest whole millimeter. Samples were performed in duplicate and results expressed as mean ± SD.

Enzyme Activity Assays

Characterization of *LfQi6* enzymatic activity was carried out with an API ZYM kit per accompanying instructions (bioMerieux, France). *LfQi6* was incubated on an MRS agar plate, and cells were suspended in 0.85% NaCl solution (McFarland turbidity adjusted to 5–6). An aliquot (65 μl) of each suspension was inoculated in each API ZYM kit cupule, and after incubating for 4 h at 37 °C, ZYM test reagent was added. Color changes were observed, and values ranging from 0 to 5 were assigned on the basis of color strength to determine the approximate amount of nanomoles (nmol) of hydrolyzed substrate. Results expressed are representative results from two independently performed experiments.

Hemolysis Assay

Hemolysis was evaluated using sheep's blood agar plates (5% defibrinated sheep's blood), incubated at 30 °C for 48 h. Recorded characteristics of hemolysis on blood agar were β-hemolysis (clear zones around colonies), α-hemolysis (green zone around colonies), and γ-hemolysis (non-hemolytic, no halo around colonies). The assay was performed in duplicate.

Biogenic Amine Production Assay

Biogenic amine production of tyramine, histamine, and putrescine was assessed using the decarboxylase agar method [15]. Precursor amino acids (tyrosine, histidine, and ornithine, respectively) were purchased from Sigma, MO. *LfQi6* was inoculated onto decarboxylase plates and incubated for 4 days at 37 °C under aerobic and anaerobic conditions. A positive result was defined as a color change of the medium from yellow to purple due to pH shift based on production of alkaline biogenic amines from the amino acids present in the medium. The assay was conducted in duplicate.

Mucin Degradation Assay

Mucin degradation was studied using 0.3% mucin-supplemented agarose medium with or without glucose (Sigma, MO) [16]. In brief, cells were grown overnight in MRS broth at 37 °C under aerobic conditions and spotted on medium B plates: tryptone (Oxoid) 7.5 g/l; casitone (Difco) 7.5 g/l; yeast extract 3.0 g/l; meat extract 5.0 g/l; NaCl (BDH) 5.0 g/l; K 2 HPO-3H 2 O (BDH) 3.0 g/l; KH 2 PO (BDH) 0.5 g/l; MgSO-7H 2 O (BDH) 0.5 g/l; cysteine HCl (Sigma) 0.5 g/l; D-(1)-glucose (BDH) 10 or 30 g/l; purified hog gastric

mucin (HGM) 3 g/l; and agarose (Sigma) 1.5 g/100 mL. The pH of medium was adjusted to 7.0 with 2 N NaOH. Mucin degradation activity was evaluated by the diameter of the halo observed after plate staining with amido black 0.1% in glacial acetic acid 3.5 M and washing with glacial acetic acid 1.2 M. Mucin used in this study was from porcine stomach type III (Sigma). A stool sample collected from a 2-month-old infant was used as a positive control.

S. enterica subsp. enterica Minimum Inhibition Concentration (MIC) Assay

A microplate plate assay was used to determine *S. enterica (Kauffman and Edwards) Le Minor and Popoff ATCC 51741* MIC. Qi6 biofilm fermented media, Qi6 planktonic fermented media, and respective autoclaved counterparts were evaluated, and 100 µL of bacteria at 3×10^8 CFU/mL was added to each well. Media alone served as negative control with meropenem as positive control (twofold dilutions of 5 mg/mL). Initial and final OD 600-nm readings were taken before and after overnight plate incubation and dilution series plated on brain heart infusion (BHI) agar and incubated overnight for standard CFU estimation the next day. Data was compared to original OD readings to determine efficacy. Negative values indicate Salmonella death. Positive values indicate Salmonella growth. Results close to zero indicate bacteriostasis. The test was performed in triplicate and results expressed as the mean ± SD.

Statistical Analysis

P values were calculated in GraphPad Prism 7 using one-way ANOVA and are represented by * $P \leq 0.05$, † $P \leq 0.01$, ‡ $P \leq 0.001$, and § $P \leq 0.0001$. Experimental data were subjected to analysis of variance (ANOVA), and multiple comparisons were performed using the Tukey–Kramer test. *P* values of < 0.05 were considered significant.

Results

In a previous study, we isolated *LfQi6* from the human microbiome for which a draft, whole-genome sequencing project was undertaken and deposited in GenBank under the accession number *LAIK00000000.1* [4]. In this study, we detail *LfQi6* phylogenetic analyses, investigate its probiotic activities, and evaluate its general safety as a probiotic.

Phylogenetic Analyses Indicates LfQi6 Evolutionary Relatedness to Reference Lactobacillus Species and *Lact. fermentum* Human Microbiome Isolates

Figure 1A depicts a phylogenetic analysis performed by aligning the 16S rRNA gene sequences from the indicated representative *Lactobacillus* species on PATRIC [17]. *LfQi6* clusters with other *Lact. fermentum* probiotic human microbiota species as well as *Lact. reuteri* which, until recently, was classified as a *Lact. fermentum* isolate [18]. Further whole-genome sequencing alignment performed on NCBI [19] shows *LfQi6* phylogenetic placement among the available *Lact. fermentum* strains for which whole or draft genome sequencing data is publicly available and its evolutionary distance from *Lact. fermentum IFO 3956* was used as the scaffold for *LfQi6* contig generation [4].

LfQi6 CFS Displays Potent Antimicrobial Effect Against Methicillin-Resistant *S. aureus* (MRSA) and *P. aeruginosa*

Broth microdilution technique was used to evaluate the ability of *LfQi6* cell-free supernatant (CFS) to inhibit the growth of two common bacterial pathogens, the antibiotic-resistant Gram-positive pathogen MRSA and Gram-negative pathogen *P. aeruginosa*. The results, depicted in Fig. 2, show potent antimicrobial activity for *LfQi6* CFS against *P. aeruginosa* (Fig. 2a) and MRSA (Fig. 2b) at 24 h, with 100% inhibition of bacterial pathogen cell proliferation in the presence of *LfQi6* CFS applied at 1:1 (*v*/v) versus PBS controls.

LfQi6 Exhibits Significant Antimicrobial Activity

The antimicrobial activities of planktonic *LfQi6* were evaluated against Gram-negative *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and Gram-positive *S. aureus* and MRSA. Zones of inhibition were measured and are presented in Table 1 showing significant antimicrobial activity against all tested pathogens.

LfQi6 Is Acid Resistant

As shown in Fig. 3a, *LfQi6* is resistant to exposure to low pH for 3 h, evidenced by its robust growth at pH 3.0 when compared to growth at physiologic pH, with minimal reduction in growth rate observed at pH 2.0.

LfQi6 Is Bile Salt Tolerant

LfQi6 bile salt tolerance was first evaluated by exposing overnight cultures to 0.3% and 2% bile acids (Oxgall) for 3 h. Total cell viability was determined by CFU plate counting (Fig. 3b). Our results indicate that *LfQi6* is tolerant to physiologically relevant concentration of 0.3% bile acid, with a reduction in cell viability observed at high bile salt concentration (2%). The inverse correlation between *LfQi6* viability and bile acid concentration was determined by titrating the indicated Oxgall concentrations into the growth medium and culturing for 24 h

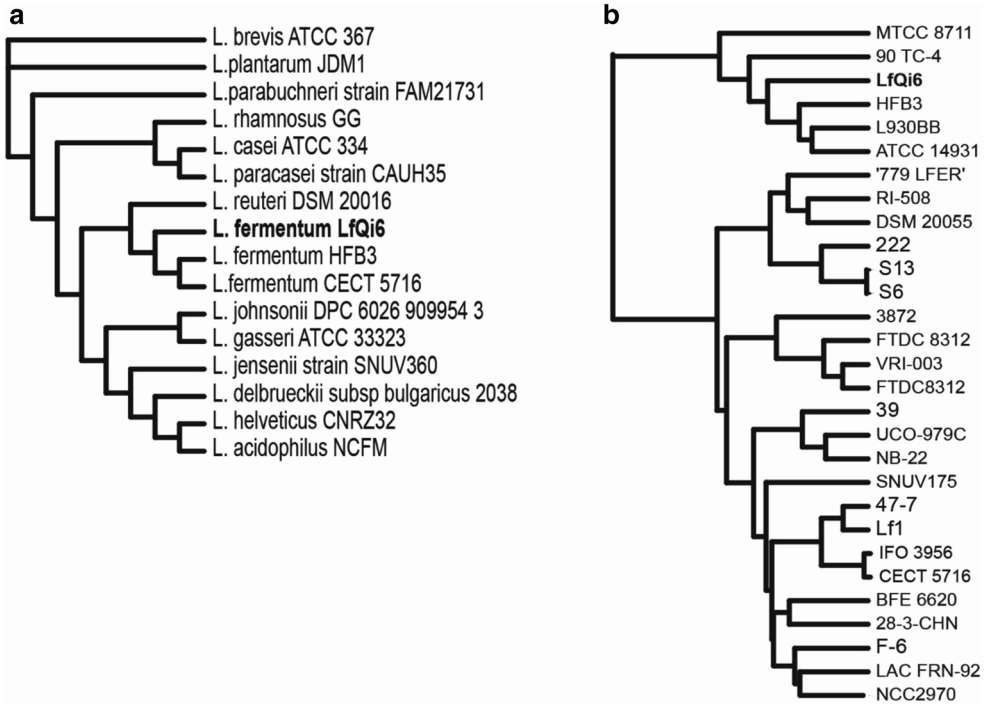

Fig. 1 Phylogenetic tree of *LfQi6* with respect to (**a**) *Lactobacillus* species and (**b**) *Lact. fermentum* strains

(Fig. 3c) and is consistent with that described in previous probiotic studies [20, 21].

LfQi6 Biofilm Formation Is Stimulated by Low pH and Bile Salts

To colonize the human gastrointestinal tract (GIT), microorganisms are primed to respond to environmental cues such as the acidic milieu of the stomach and the bile acid-rich upper intestinal compartments, through a transition from planktonic to a biofilm mode of cell metabolism. Figure 3d shows that this transition to biofilm growth phenotype occurs as *LfQi6* which encounters low pH and bile salts.

LfQi6 Biofilm Cell Mass vs LfQi6 Planktonic Cell Mass Shows Increased Bile Salt Hydrolase (BSH) Activity

Bile salt tolerance requires hydrolase activity. Bile salt hydrolases (EC 3.5.1.24) deconjugate bile salts by hydrolyzing the amide bond to release the glycine/taurine moiety, resulting in deconjugated bile acids. Based on metabolic reconstruction and strain analysis using Rapid Annotations using Subsystems Technology (RAST), *LfQi6* encodes two bile salt hydrolases, the activity of which can be observed in Fig. 3e and is enhanced during biofilm growth as a white precipitate surrounding the colony [22].

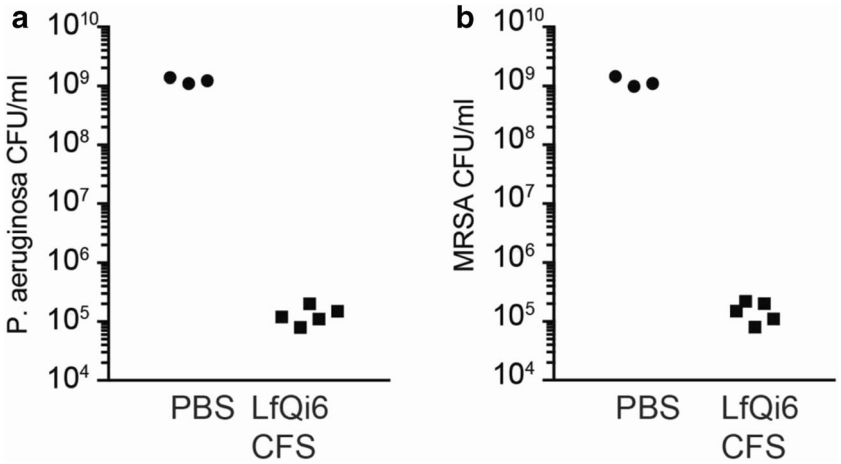

Fig. 2 *LfQi6* cell-free supernatant exhibits potent antimicrobial activity against representative Gram-negative and Gram-positive pathogens (**a**) *Pseudomonas aeruginosa* and (**b**) methicillin-resistant *S. aureus* (MRSA)

Table 1 *LfQi6* exhibits broad antimicrobial activity against Gram-negative and Gram-positive human pathogens

Pathogen	Inhibition zone (mm from colony edge to clear zone)
Escherichia coli K12 (ATCC 25404)	12
Klebsiella pneumoniae (clinical strain)	6
MRSA (ATCC 33591)	8
Pseudomonas aeruginosa (clinical strain)	7
Staphylococcus aureus (ATCC 25923)	4

LfQi6 and the indicated pathogens were grown overnight and soft top MRS agar inoculated with the indicated pathogens was dispensed on solid MRS agar plates and *LfQi6* spotted onto the solidified soft top agar inoculum. Zones of inhibition were measured to the nearest whole millimeter. Plates were performed in duplicate

Cholesterol Assimilation by LfQi6

To assay for *LfQi6* cholesterol-lowering ability, overnight cultures were grown in the presence of 0.2% thioglycolate, 0.3% Oxgall (to stimulate BSH activity), and water-soluble cholesterol. Cholesterol concentration in cell-free medium was determined after 17 h of growth. As can be seen in Fig. 3f, *LfQi6* reduced cholesterol levels in the media by approximately 30% versus a no-bacteria experimental sample included as a control for any effects of the culture medium on cholesterol hydrolysis.

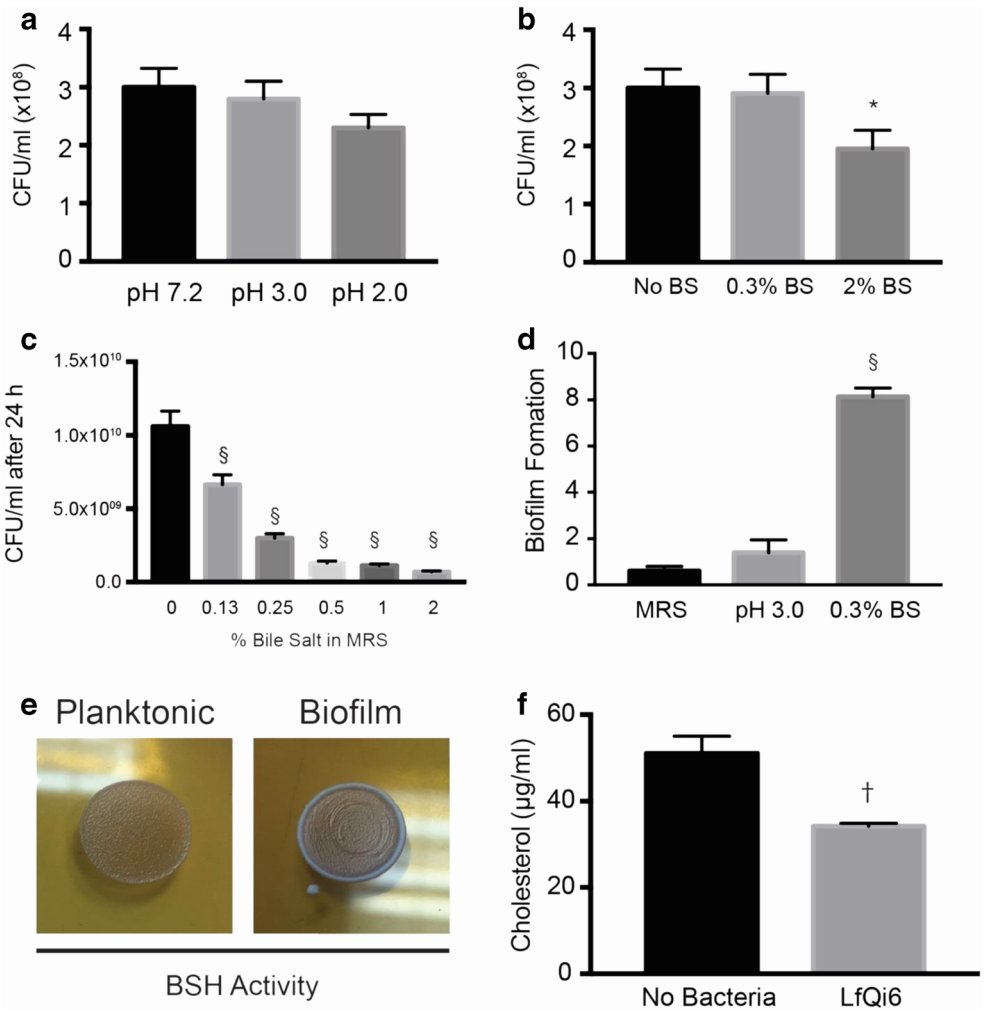

Fig. 3 *LfQi6* shows (**a**) acid resistance at pH 2.0. *LfQi6* was grown in MRS broth and approximately 10^6 CFUs exposed to the indicated pH for 3 h before assessing viability by CFU plating. (**b**) Bile salt tolerance. *LfQi6* was grown in the presence of the indicated concentrations of Oxgall bile salts for 24 h. CFUs were enumerated to determine viability. (**c**) Dose-dependent bile salt viability. *LfQi6* viability was observed under the indicated concentrations of Oxgall bile salts after 24 h. (**d**) *LfQi6* biofilm formation is stimulated by low pH and bile acids. (e) *LfQi6* bile salt hydrolase activity is enhanced in *LfQi6* biofilm cellular mass. (**f**) *LfQi6* assimilates cholesterol as evidenced by a decrease in media cholesterol concentration when compared to the cell-free control media. *P* values calculated in GraphPad Prism 7 using one-way ANOVA and are represented by * $P \leq 0.05$, † $P \leq 0.01$, ‡ $P \leq 0.001$, and § $P \leq 0.0001$

LfQi6 Biofilm Cell Mass vs LfQi6 Planktonic Cell Mass Shows Superior Survival in Simulated Fasted and Fed Human Intestinal Fluids

Additional testing using a biorelevant media to simulate the fasted and fed states of the human intestine was used to evaluate survival of *LfQi6* during gastrointestinal transit (Fig. 4). Reaching statistical significance ($P \leq 0.0001$), biofilm cultures demonstrated improved survival by approximately 40–50% over planktonic cultures when exposed to fed and fasting intestinal conditions. Neither culture was able to sustain growth in simulated fasting gastric media.

LfQi6 Displays High Cell Surface Hydrophobicity (CSH) vs LGG

To determine *LfQi6* CSH, the ability of an overnight inoculum, resuspended in PBS, to move into a hydrophobic hydrocarbon (xylene) phase was tested. As shown in Fig. 5a, *LfQi6* exhibits very high CSH, with almost 60% of the initial inoculum retained in the organic phase. In contrast, *LGG* displays a significantly lower CSH of 18% (†$P \leq 0.01$).

LfQi6 Auto-Aggregates

To measure auto-aggregation, overnight cultures resuspended in PBS (OD_{600} 0.3) were incubated without shaking at 25 °C. Optical density was measured at 3, 5, and 24 h and percent auto-aggregation determined. Figure 5b describes the increase in *LfQi6* auto-aggregation over time, with almost 60% auto-aggregation observed after 24 h of static incubation († $P \leq 0.01$).

LfQi6 Co-aggregates with *E. coli* and MRSA

The ability of *LfQi6* to co-aggregate with Gram-negative *E. coli* and Gram-positive MRSA was determined spectrophotometrically over 3, 5, and 24 h of static growth. Results show significant *LfQi6* co-aggregation with *E. coli* and MRSA after 3 h of co-culture, at approximately 30% and 40%, respectively (Fig. 5c). This is further supported by the microscopic visualization of *LfQi6*-pathogen aggregates at the same time point (Fig. 5d).

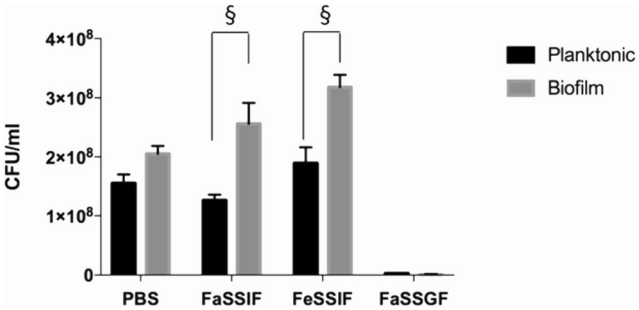

Fig. 4 Biofilm culture demonstrates superior survivability over planktonic culture in simulated fasting and fed human intestinal fluids (FaSSIF, Fasting State Intestinal Fluid; FeSSIF, Fed State Intestinal Fluid; FaSSGF, Fasting State Gastric Fluid). *P* values calculated in GraphPad Prism 7 using one-way ANOVA and are represented by * $P \leq 0.05$, † $P \leq 0.01$, ‡ $P \leq 0.001$, and § $P \leq 0.0001$

LfQi6 Biofilm Cell Mass vs LfQi6 Planktonic Cell Mass Binds Efficiently to Human Gastrointestinal Cell Monolayer

LfQi6 adheres to differentiated human Caco-2 cells, comparable to that of *LGG* († $P \leq 0.01$) (Fig. 6a). *LfQi6*-Caco-2 adhesion efficiency increased when *LfQi6* cells derived from biofilm cellular mass versus planktonic cells were used (Fig. 6b). This binding efficiency difference is pictorially represented by microscopic visualization of both *LfQi6* planktonic cells (left panel) and those derived from biofilm cellular mass (right panel), showing a significantly higher number of biofilm-derived *LfQi6* cells bound to polarized Caco-2 cells (Fig. 6c).

LfQi6 Biofilm Cell Mass vs LfQi6 Planktonic Cell Mass Has Significant Feruloyl Esterase Activity (FEA) vs LGG (None)

LfQi6 shows robust FEA as can be seen in Fig. 7 (top left panel), the halo representing the hydrolysis of methyl ferulate. In contrast, *LGG*, for which a gene encoding FEA has not been found, is unable to hydrolyze the FEA substrate within the detection levels of this assay (top right panel). Casein protease activity serves as a control for cell density and viability as well as functional bacterial cell secretory processes (lower panels). No significant differences were found between casein protease activity and approximate cell density/viability.

LfQi6 Biofilm Cell Mass Has Higher Glutathione Content and Glutathione:Glutathione Disulfide Ratios (GSH:GSSG) than LfQi6 Planktonic Cell Mass

Glutathione is a prominent cellular antioxidant and important cofactor in detoxification of reactive oxygen species (ROS). Intracellular total, free, and oxidized GSH levels were measured in *LfQi6* biofilm and planktonic cell mass and compared against bacterial media. Because *LGG* is incapable of GSH synthesis, *LGG* was used as a negative probiotic control for GSH accumulation and/or secretion [23]. Results support *LfQi6* GSH autotrophy, with threefold higher total GSH levels in *LfQi6* culture media versus *LGG* (§ $P \leq 0.0001$) (Fig. 8a), and there is no significant increase in total GSH in *LfQi6* growth media. The observation of altered GSH:GSSG ratios in the media and cells

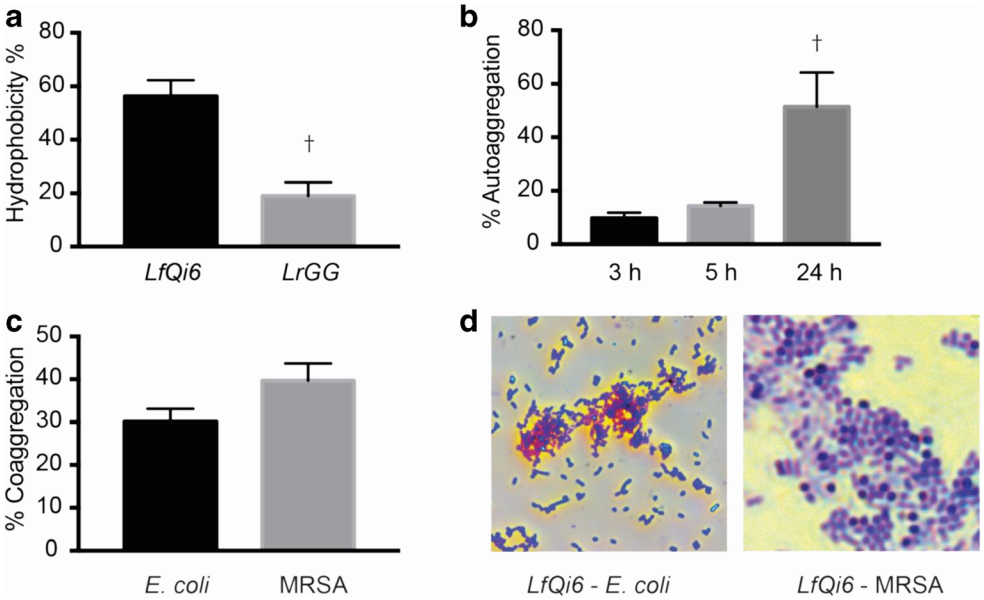

Fig. 5 *LfQi6* adhesive properties. *LfQi6* shows (a) high CSH, (**b**) auto-aggregation and (**c**) co-aggregation with *E. coli* and MRSA. (d) Microscopic visualization of *LfQi6*-pathogen aggregates. Light blue rods – *LfQi6*; dark blue cocci – MRSA; pink rods – *E. coli* (left panel, *E. coli*; right panel, MRSA). *P* values calculated in GraphPad Prism 7 using one-way ANOVA and are represented by * $P \leq 0.05$, † $P \leq 0.01$, ‡ $P \leq 0.001$, and § $P \leq 0.0001$

of planktonic and biofilm *LfQi6* indicates the probability of active bacterial GSH import/export (Fig. 8a and b). *LfQi6* cells grown as a biofilm cellular mass show significantly higher levels of secreted total GSH when compared to planktonic cells, with a significantly increased GSH:GSSG ratio vs *LfQi6* planktonic cellular mass.

LfQi6 Biofilm Cell Mass But Not LfQi6 Planktonic Cell Mass Contributes GSH to Caco-2 Monolayer

Differentiated Caco-2 cells (5×10^4) and *LfQi6* planktonic or biofilm cell mass (1×10^9) were grown overnight in co-culture to model the probiotic host cell paradigm, and the GSH:GSSG

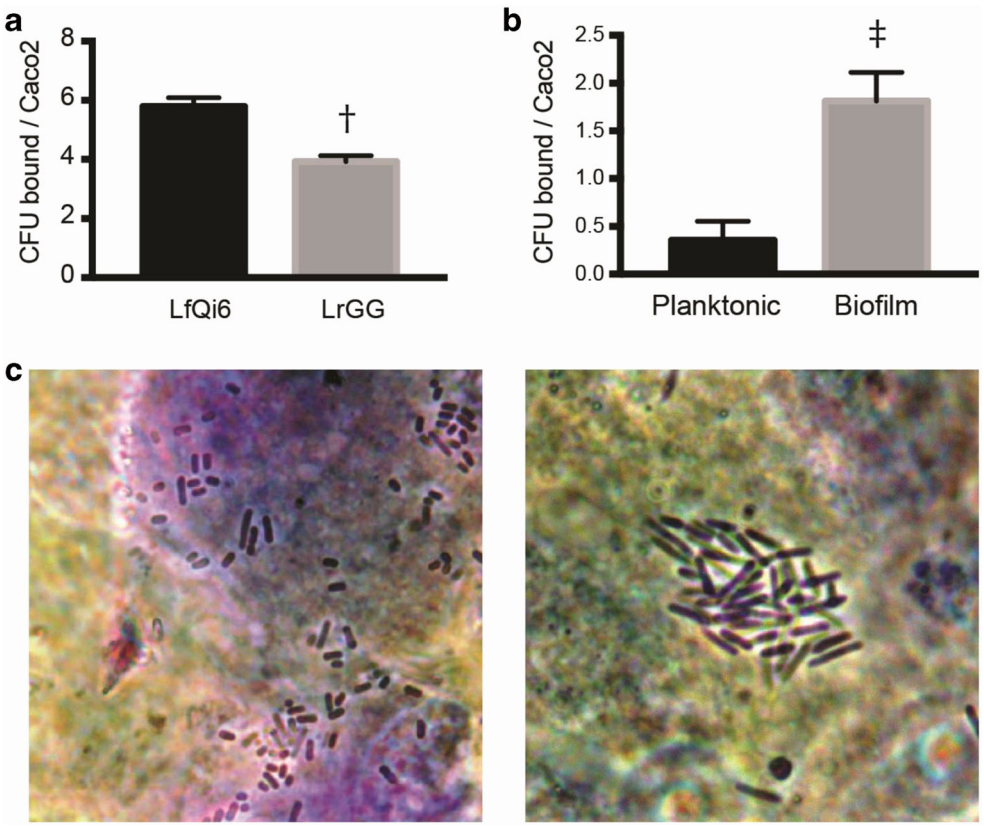

Fig. 6 *LfQi6* significantly binds to human differentiated Caco-2 cells. (**a**) *LfQi6* binds to Caco-2 cells at levels comparable to *Lact. rhamnosus GG*. (**b**) Biofilm-derived *LfQi6* shows enhanced adhesion to Caco-2 cells when compared to planktonic *LfQi6*. (**c**) Microscopic visualization of adhered planktonic (left panel) and biofilm-derived *LfQi6* (right panel) to Caco-2 cells. *P* values were calculated in GraphPad Prism 7 using one-way ANOVA and are represented by * $P \leq 0.05$, † $P \leq 0.01$, ‡ $P \leq 0.001$, and § $P \leq 0.0001$

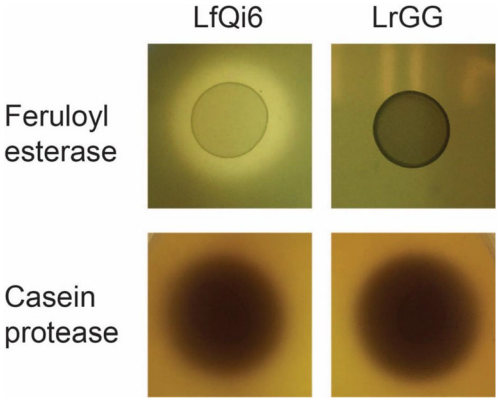

Fig. 7 *LfQi6* expresses and secretes feruloyl esterase (FEA). In contrast, *LrGG*, which does not encode FEA, does not display the ability to hydrolyze methyl ferulate. Casein protease activity was included as a "loading" control relative to approximate cell number and viability (as indicated by opacity and density) as well as a functional cell secretory system under the conditions tested

ratio was determined in the Caco-2 cell culture media and in Caco-2 cells. While no differences were found in the GSH:GSSG ratio in Caco-2 media inoculated with PBS and planktonic *LfQi6*, there was a statistically significant decrease in GSSG levels in media derived from Caco-2-*LfQi6* biofilm co-culture (Fig. 8c). Similarly, the GSH:GSSG ratio is unchanged in Caco-2 cells co-cultured with planktonic *LfQi6*, as compared to the PBS control. However, there is striking increase in total and free GSH in Caco-2 cells co-cultured in with *LfQi6* biofilm cell mass, indicative of its unique contribution and availability to Caco-2 cells vs its planktonic counterpart.

LfQi6 Biofilm Cell Mass But Not LfQi6 Planktonic Cell Mass Increases Total Antioxidant Capacity (TAC) of Caco-2 Monolayer

Total antioxidant capacity (TAC) measures the antioxidant status of biological samples and cells and is indicative of the capacity to evoke an antioxidant response against free

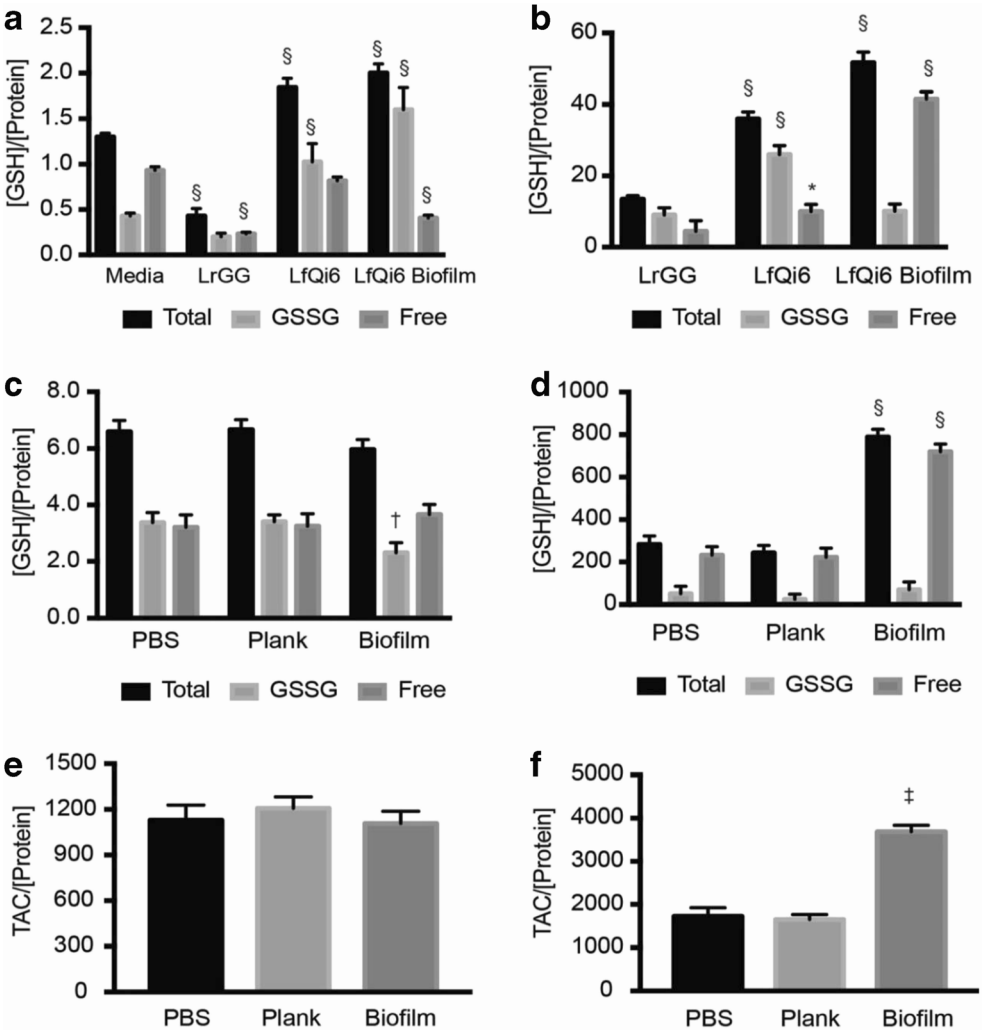

Fig. 8 *LfQi6* is a significant source of reduced GSH and increases the total antioxidant capacity (TAC) of mammalian cells. Total, reduced (free), and oxidized glutathione was quantified in (**a**) the growth media of *LfQi6* grown as planktonic cells or biofilm cellular mass (**b**) *LfQi6* cells grown as planktonic cells or biofilm. (**c**) Caco-2 cell culture media. (**d**) Caco-2 cells. Total antioxidant capacity (TAC) was determined for (**e**) *LfQi6* planktonic cell mass vs biofilm cell mass fraction and (**f**) Caco-2 cells. *LrGG* was included as a probiotic reference. *P* values were calculated in GraphPad Prism 7 using one-way ANOVA and are represented by * $P \leq 0.05$, † $P \leq 0.01$, ‡ $P \leq 0.001$, and § $P \leq 0.0001$

radicals. TAC determination encompasses all antioxidants including glutathione and dietary antioxidants such as vitamins A, C, and E and enzymes including catalase and superoxide dismutases. Results show that TAC remains unchanged in Caco-2 growth media when co-cultured with planktonic or biofilm cell mass *LfQi6* (Fig. 8e). Strikingly, a dramatic increase in Caco-2 cell TAC is observed under *LfQi6* biofilm cell mass-Caco-2 co-culture but not with *LfQi6* planktonic or control co-cultures (Fig. 8f). Together, these data highlight the antioxidant benefits to mammalian cells uniquely conferred by *LfQi6* biofilm cell mass.

LfQi6 Demonstrates High Enzyme Activities Potentially Beneficial to Human Health

LfQi6 exhibits high enzyme activities for enzymes potentially beneficial to the human host (Table 3). For instance, *LfQi6* demonstrates high activity for β-galactosidase, greater than *LGG*, at ≥ 30 nmol of substrate hydrolyzed. The microbial β-galactosidase enzyme has shown potential for treatment of lactose intolerance [25] as well as the metabolism of bifidogenic, prebiotic galactooligosaccharides, present, for instance, in human breastmilk [26]. Additionally, *LfQi6* has high α-galactosidase activity, ≥ 40 nmol of substrate hydrolyzed. Oral supplementation with a highly active α-galactosidase *Lact. fermentum* strain improved abdominal symptoms due to colonic microbial prebiotic fermentation of widely consumed legumes such as soy and beans, as this enzyme is lacking in mammals [27].

LfQi6 Biofilm But Not Planktonic Media Is Inhibitory Against *S. enterica*

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial ingredient or agent that is bacteriostatic. Whereas the planktonic media was entirely ineffective, both autoclaved and nonautoclaved biofilm media demonstrated effective MICs up to ¼ dilution factor (Fig. 9). The active factors responsible for this effect are heat-stable, as autoclaving the media maintains this effect. Moreover, this effect is not pH-dependent, as the pH of the biofilm and planktonic spent medias are essentially the same (data not shown).

LfQi6 Demonstrates No In Vitro Safety Concerns: Biogenic Amine Production, Mucin Degradation, Hemolysis, Antimicrobial Resistance Pattern, and Enzymatic Activity Evaluations

A standard probiotic safety evaluation, via antimicrobial resistance pattern determination and assessment for potentially harmful metabolic activities, such as biogenic amine production, mucin degradation, various enzymatic activities, and pathogenic hemolytic activity, was undertaken. None of these evaluations demonstrate any safety concerns. The minimum inhibitory concentration (MIC) of antibiotics of the isolates

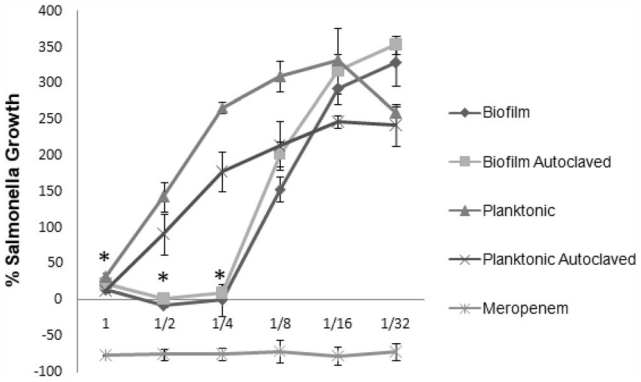

Fig. 9 *LfQi6* autoclaved and non-autoclaved biofilm medias but not planktonic medias are capable of inhibiting Salmonella growth similar to meropenem. An aliquot (100 μL of 3×10^8 CFU/mL) of *S. enterica (Kauffman and Edwards) Le Minor and Popoff ATCC 51741* was incubated overnight in each well of a microplate assay with respective media components and controls as indicated (positive control, twofold dilutions of meropenem at 5 mg/mL with media alone as negative control). Initial and final OD 600-nm readings were taken before and after incubation. Dilution series was plated on BHI agar for CFU estimation the following day. The test was performed in triplicate and results expressed as the mean ± SD. *P* values were calculated in GraphPad Prism 7 using one-way ANOVA and are represented by * $P \leq 0.05$, † $P \leq 0.01$, ‡ $P \leq 0.001$, and § $P \leq 0.0001$

Table 2 Antibiotic susceptibility testing for LfQi6

Antibiotics	Dose	Planktonic sensitivity	Basis of resistance
Amoxi-Clav	20/10	S	
Ampicillin	10	S	
Cefoxitin	30	R	Intrinsic
Chloramphenicol	30	S	
Ciprofloxacin	5	S	
Clindamycin	2	S	
Daptomycin	30	S	
Erythromycin	15	S	
Fosfomycin	200	R	Intrinsic
Gentamicin	10	R	Intrinsic
Imipenem	10	S	
Linezolid	30	S	
Meropenem	10	S	
Oxacillin	1	S	
Penicillin (Units)	10	S	
Rifamopin	5	S	
Tetracycline	30	S	
SMZ-TMP	5	R	Intrinsic
Vancomycin	30	R	Intrinsic

LfQi6 shows intrinsic antibiotic susceptibility characteristic of probiotic LAB. S = sensitive; R = resistant. Doses in micrograms except where noted

was tested using the antibiotic panel recommended by EFSA [14, 28] (Table 2). *LfQi6* displays antibiotic susceptibility typical of a generally recognized as safe (GRAS) LAB strain, with only the intrinsic resistance pattern expected for lactobacilli observed, with resistance to cefoxitin, fosfomycin, gentamycin, sulfamethoxazole, and trimethoprim (SMZ-TMP) and vancomycin.

In an evaluation for various enzymatic activities, *LfQi6* showed no concerning activities (Table 3). *LfQi6* was negative for β-glucosidase, *N*-acetyl-β-glucosaminidase, and β-glucuronidase. With exception of β-glucosidase, *LGG* is also negative for these activities. Like *LGG*, *LfQi6* does not possess amino acid decarboxylase activity capable of generating potentially harmful biogenic amines such as histamine and tyramine and is also negative for protease activity, as demonstrated by non-reactivity in casein plate testing. Moreover, *LfQi6* is non-hemolytic and non-mucinolytic (Table 4). These results agree with previous *Lact. fermentum* evaluations (FDA GRN No. 531 *Lact. fermentum CECT5716*). In summary, these in vitro results do not raise any safety concerns for probiotic use of this particular *Lact. fermentum* strain.

Table 4 *LfQi6* is non-hemolytic and negative for biogenic amine production and mucin degradation

Safety aspect	
Hemolysis	Gamma
Biogenic amine production (ornithine, lysine, histidine, tyrosine)	Negative
Mucin degradation	Negative

Discussion

The World Health Organization (WHO) has defined probiotics as "live micro-organisms which when administered in adequate amounts confer a health benefit on the host" [1]. While it is well-known that the efficacy of probiotics is strain-specific, less well-known are the potentially significant impacts of organism growth conditions, such as biofilm versus planktonic phenotype, on probiotic benefit profile and efficacy. The potentially significant impact of microbial biofilms on probiotic research and development may be based on traits intrinsic to biofilms themselves. Biofilm characteristics contrast strikingly with those of planktonic organisms, which are freely mobile microbes functioning alone, consequently vulnerable to chemical, microbial, and host attack [29]. Biofilms function as polymicrobial consortia attached to living or inert surfaces. One particularly important characteristic of these niche-dwelling communities is their secretion of an extracellular matrix which envelopes and embeds the microbial colonies. This physical matrix is important for the efficient adhesion to human body surfaces, physical maintenance of the microbial colonies, concentration and diffusion of nutrients,

Table 3 *LfQi6* fermentative and metabolic enzyme activity

Enzymes	*Lact. fermentum LfQi6*	*Lact. rhamnosus* GG
Alkaline phosphatase	0	4
Esterase	2	3
Esterase lipase	2	3
Lipase	0	0
Leucine arylamidase	4	5
Valine arylamidase	2	3
Cysteine arylamidase	3	4
Trypsin	0	0
α-chymotrypsin	0	1
Acid phosphatase	1	5
Naphthol-AS-BI-phosphohydrolase	5	5
α-galactosidase	5	1
β-galactosidase	4	3
β-glucuronidase	0	0
α-glucosidase	4	3
β-glucosidase	0	5
N-acyl-β-glucosaminidase	0	0
α-mannosidase	0	0
α-fucosidase	0	5

Note: Enzyme activity range of 0 to 5 = 0, no activity; 1, 5 nmols; 2 = 10 nmols; 3 = nmols; 4 = 30 nmols; 5 = 40 nmols of APIZYM substrate hydrolyzed during 4-h incubation [24]. Reactions of > 3 (20 nmols) were considered strongly positive; 1 and 2 were considered weakly positive

toxin exclusion, inhibition of host immune response, prevention of microbial invasion, and enablement of chemical communication within the biofilm community, called quorum sensing [6].

This biofilm phenotype is by its nature uniquely advantageous to microbial survival, perhaps particularly on the hostile landscapes of the human body. While there has been more research on pathogenic biofilms [4], less work has been done on commensal biofilms. However, commensal bacteria also have adapted to life on human intestinal tract or skin by adhering to and populating these generally antimicrobial surfaces as microbial biofilms [30, 31]. Based on the concept of evolutionary symbiosis between the human microbiome and human physiology, we cultured commensal human microbiota, in this case, *Lact. fermentum LfQi6*, for evaluation as probiotics. Furthermore, we hypothesized that LAB preformed biofilms may represent enhanced probiotic delivery systems potentially more likely to colonize human body surfaces and that these more "physiologic" preformed biofilm biomass LAB may have additional, unknown probiotic benefits to the human host unique to their biofilm phenotype. To test our hypothesis that probiotic benefits and/or delivery of those benefits may be potentiated by the use of LAB preformed biofilms, we isolated *LfQi6* biofilm cell mass fractions to compare certain probiotic characteristics against planktonic cellular samples.

Antimicrobial activities of LAB are an initial defining probiotic property. Antimicrobial strategies common to probiotic bacteria include the biosynthesis and secretion of antimicrobial peptides and molecules, biosurfactants and acidification of the extracellular milieu, as well as cell-intrinsic activities that modulate pathogen fitness, adhesion, and dispersal. We tested the ability of *LfQi6* cell-free supernatant to inhibit the growth of two leading causes of nosocomial infections, *P. aeruginosa* and MRSA. *LfQi6* cell-free supernatant generated from *LfQi6* biofilms completely inhibited these pathogens; notably, planktonic bacterial components did not. The activity of this particular preformed commensal biofilm against these two pathogens in the ESKAPE family (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), as well as against an increasingly prevalent intestinal pathogen, *S. enteritidis*, suggests a potential alternative approach to the treatment of pathogens and antimicrobial resistance via the use of commensal biofilms and their components. For instance, *LfQi6* biofilm generates a heat-stable, pH-independent antimicrobial factor which could be utilized in hygiene or disinfectant products to block attachment of, inhibit, or even remove, pathogenic biofilms.

We further examined other probiotic properties of *LfQi6*, including its potential to survive gastrointestinal passage. Survival of probiotic bacterial strains in the intestinal tract is critical to probiotic efficacy. The ability of bacterial strains to survive in vitro simulated conditions of the gastrointestinal tract is used to predict in vivo viability [32]. Therefore, in vitro ability of *LfQi6* to survive gastrointestinal transit was examined at simulated gastric acidity of pH 2 and 3. To be considered probiotic, the microorganism must withstand pH 3.0 for 3 h, simulating survival at physiologic gastric conditions [33, 34]. *LfQi6* showed excellent viability at low pH, indicating high likelihood of survival through gastric transit. Indeed, sequencing analysis has shown *LfQi6* that possesses genes which have been shown by others to confer acid resistance, namely, proton extruding F_0F_1-ATPase and increased expression of the chaperone genes dnaK, groEL, clpB, and clpE, among others [1]. Finally, *LfQi6* biofilm cell mass showed superior survivability in simulated fasted and fed intestinal tract fluid when compared against *LfQi6* planktonic sample.

Following successful transit through the stomach, probiotic organisms must then survive duodenal bile salt-mediated bacterial cell membrane emulsification and oxidant stress. Due to bile acid-mediated bacterial toxicity, gut biliary acids essentially help shape the gut microbial profile by selecting microbiota capable of surviving biliary acid exposure [35]. Results in this report indicate that *LfQi6* is multifactorially adapted to physiologically relevant bile acid concentrations, through its glutathione synthesis machinery as well as its bile salt hydrolase enzymes [36]. Additionally, because *LfQi6* biofilm cell mass appears uniquely able to donate antioxidant stores to the surrounding milieu, *LfQi6* biofilm cell mass administered as a probiotic fraction in vivo may deliver glutathione stores to human host tissues.

LfQi6 bile salt metabolism may be enhanced in preformed biofilms. The bacterial bile salt hydrolase enzyme (BSH) deconjugates bile salts, protecting bacteria [37]. Increased copy number of BSH isoforms in LAB confers even greater protection [38] LfQi6 genomic analysis demonstrates two BSH enzymes, as well as several α- and β-hydrolases previously shown to metabolize bile salts (Table 3). Microbial BSH activity may benefit its host by decreasing cholesterol absorption, a recognized health benefit for the management of cardiovascular disease [39, 40]. Hence, *LfQi6* microbial BSH activity may be considered a probiotic trait [41]. Interestingly, BSH activity increases significantly in *LfQi6* biofilm cell mass when compared to *LfQi6* planktonic cells. As it is thought that the decomposition of bile salts by the BSH enzyme disrupts the formation of the cholesterol micelle, thereby preventing host cholesterol absorption [42], it is tempting to speculate that the increased BSH activity of *LfQi6* biofilm cell mass may translate into clinically improved cholesterol lowering effect when using the *LfQi6* preformed biofilm cell mass rather than planktonic cells. Importantly, the benefit of bile salt tolerance appears to be specific to both

strain and biofilm phenotype, as other *Lact. fermentum* strains grown as biofilms cannot grow under the same conditions [43].

Another bacterial adaptation to bile salt exposure and other gastrointestinal hostilities is the microbial antioxidant stress response [44]. There is increasing evidence of beneficial antioxidative effects from probiotic lactic acid bacteria on human health [45–47]. The small molecule glutathione (GSH; L-gamma-Glu-L-Cys-Gly) is the main nonenzymatic antioxidant in eukaryotic cells, responsible for detoxifying reactive oxygen species (ROS), reducing glutaredoxins (small antioxidant enzymes), deconjugating xenobiotics, transporting and storing nitric oxide (NO), and other cellular functions, such as immune support [48]. Glutathione is active in its reduced state, cycling between its reduced, free form (GSH) to a disulfide oxidized (GSSG) form and to buffer and maintain cellular redox homeostasis [49], critical to cell and organism viability [50]. While ubiquitous to Gram-negative bacteria, GSH is rarely found in Gram-positive bacteria, in which it has only recently been described [51]. However, a complete GSH system has been described in *Lact. fermentum* [52].

In experiments described in this report, *LfQi6* appears unique in its ability to synthesize GSH and donate this potent antioxidant to its host. Results indicate significantly elevated GSH levels in *LfQi6* culture media when compared against LGG (a strain unable to synthesize GSH) and media alone, indicating that *LfQi6* synthesizes GSH, actively secreting this antioxidant to the extracellular space, where it is then available for utilization by mammalian cells. Moreover, this glutathione-antioxidant "donor effect" is potentiated by the utilization of *LfQi6* biofilm cellular mass: results indicate significantly higher levels of GSH secreted by *LfQi6* biofilm cell mass, underscored by the striking difference in GSH:GSSG ratio observed in biofilm vs planktonic cell mass (Fig. 7b). The GSH:GSSG ratio favoring biofilm cell mass may reflect heavier planktonic microbial intracellular GSH consumption due to the increased metabolic demands of planktonic bacterial cells relative to the "resting metabolism" of biofilm cells, in which many cells grow slowly or not at all [53, 54], as well as biofilm adaptation to a generally more oxidative environment. Furthermore, unlike many other probiotics, *LfQi6* does not deplete host GSH levels but instead increases the antioxidant status of human tissues, as indicated by the significant increase in total antioxidant capacity of human cell monolayer when treated with *LfQi6* biofilm cellular mass vs planktonic cellular mass (Fig. 7f). These results indicate *LfQi6*-derived extracellular GSH is donated and taken up by human tissues, where it exerts a significant probiotic antioxidant effect potentiated through the use of biofilm cellular mass.

After surviving conditions in the acidic stomach and biliary salts of the duodenum, potential probiotic strains must adhere to the intestinal lining for colonization and engraftment, in order to establish probiotic host benefits [55, 56]. Cell surface hydrophobicity (CSH) is often considered a critical parameter influencing the strength of microbial host adhesion and colonization of mucosal surfaces [57]. Probiotics can inhibit mucosal pathogens either by forming a barrier via auto-aggregating or by directly co-aggregating with the pathogen [58, 59]. Therefore, co-adhesion and aggregation abilities are often considered probiotic traits. Furthermore, the degree of auto-aggregation and hydrophobicity are considered proportional to the ability of the isolates to adhere to and colonize the gastrointestinal system [24]. The high CSH observed for *LfQi6* and its ability to form auto-/co-aggregates are strongly predictive of its ability to adhere to human cells.

Consistent with demonstration of *LfQi6* high CSH, aggregation, and co-aggregation, both genetic analysis of *LfQi6* and human intestinal monolayer adhesion tests demonstrate additional evidence for *LfQi6* microbial adhesion and colonization of the human gastrointestinal system. *LfQi6* shows significant adhesion to differentiated human intestinal Caco-2 cells at a level comparable to that of the commonly referenced probiotic, LGG (Fig. 5a). Indeed, results show improved Caco-2 adhesion for *LfQi6* biofilm vs planktonic cell mass (Fig. 5b and c). Additional genomic analysis of *LfQi6* provides strain-level evidence for host gastrointestinal adhesion via demonstration of host-binding mucosal-surface mucin glycoproteins. In silico analyses of the *LfQi6* draft genome sequence reveals known and putative adhesins, including, importantly the identification of a conserved mucin-binding protein, LAIK01P001751. This protein belongs to the MUB/MUC family of bacterial mucin adhesion factors which contain the YSIRK secretion signal and sortase-dependent LPXTG anchor motif. It is tempting to speculate that the potentiation of microbial host binding which occurs when human intestinal monolayer is treated with *LfQi6* biofilm cellular mass fraction may be due to the increased expression of atypical mucus-binding proteins such as GroEL and Ef-TU [60].

Microbial enzymes in the human gut transform foods into biologically active, beneficial compounds. For instance, dietary plant compounds are metabolized in the human gastrointestinal system into bioavailable and physiologically significant antioxidant phenols via the enzyme feruloyl esterase (FEA), also known as cinnamoyl esterase (CEA). FEA hydrolyzes hydroxycinnamate esters in cereals, fruits, and vegetables to release hydroxycinnamic acids (HA), such as ferulic, sinapic, caffeic, and p-coumaric acids [61]. Ferulic acid (FA) is a powerful antioxidant and induces important host antioxidant responses, such as superoxide dismutase, catalase, and glutathione reductase, and has shown protection against diseases such as cancer, diabetes, heart disease, and Alzheimer's disease [62, 63]. Significantly, FEA is both microbial and mammalian in origin [64]. Because the benefits of FEA are proportional to its bioavailability, gastrointestinal microbial FEA to increase absorbable FEA levels is considered an important probiotic trait [65]. Indeed, animal model evidence

indicates that host augmentation with FEA-positive strains is beneficial: supplementation with FEA-positive *Lact. fermentum* improves systemic oxidant status and metabolic markers in dysmetabolic and diabetic mice [66, 67]. While FEA is not widespread among human bacterial microbiota [68], *LfQi6* genome analysis predicts a FEA gene, and qualitative plate FEA assay demonstrates FEA for the *LfQi6* strain. Moreover, FEA is notably potentiated in *LfQi6* biofilm cell mass. When considered together with the *LfQi6* biofilm cell mass-potentiated glutathione donor effect, it appears that these important, synergistic antioxidant probiotic traits could most effectively be delivered to the host GI tract as preformed *LfQi6* biofilms. Finally, a standard probiotic safety evaluation, including determination of antimicrobial resistance patterns and assessment for metabolic activities potentially harmful to the host, was undertaken and revealed no concerns.

These in vitro studies strongly support the notion that *LfQi6* elicits physiologically relevant adhesion properties required for host GI colonization and engraftment, particularly when *LfQi6* is administered as a preformed biofilm cell mass. The use of the *LfQi6* biofilm cell mass demonstrates significantly potentiated activity for the enzymes feruloyl esterase (FEA) and bile salt hydrolase (BSH) and increased glutathione production and GSH donation to host cells, with accompanying potentiated antioxidant protection. These results support the novel concept that the use of preformed *LfQi6* biofilm cell mass and potentially other preformed probiotic biofilm cells may significantly improve host delivery of probiotic benefits, as opposed to the current standard planktonic probiotic culture paradigm. Additionally, while most biofilm research has focused on pathogens, results reported here suggest a unique symbiosis for probiotic commensal biofilms and host cell colonization which may be important to take into early consideration in probiotic evaluation and development.

Conclusions and Potential Applications

The recent and intense focus on the human microbiome has inspired research into the identification of uniquely efficient and beneficial probiotics. Although biofilms are traditionally associated with virulence and chronic infection, biofilm formation is a trait common to pathogens and commensals alike. More is known about probiotic anti-biofilm activities against pathogens, such as the inhibition by various lactobacilli of biofilm-forming cariogenic *S. mutans* [69] or quorum-sensing bacteriocins produced by lactic acid bacteria [70]. Although still very little is known about the roles of probiotic biofilms, this report contains our evaluation of one such identified human commensal bacterium, *Lact. fermentum* strain, *LfQi6*. Because *LfQ6* is intended for use as a probiotic, the safety of this strain was evaluated based on published regulatory guidelines of the EFSA (QPS) and the Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food. This safety assessment included a series of in vitro experiments to characterize the new microbial strain and assess its potential toxicity. Furthermore, because the hypothesis evaluated in this communication was whether probiotic microbiota cultured as biofilms which could demonstrate unique and/or potentiated human health benefits versus same-strain planktonic culture, this assessment also included in vitro experiments to assess the influence on strain-specific probiotic traits of two differently generated samples, namely, *LfQi6* biofilm cell mass versus *LfQi6* planktonic cell mass.

The strain *LfQi6* was initially identified at the species and strain level using whole-genome sequencing as well as standard microbiological biochemical and phenotypic techniques. This identification satisfies QPS status defined by EFSA for the *Lact. fermentum* species. Evaluated according to EFSA guidelines [14], the strain's antibiotic resistance demonstrated typical intrinsic resistance patterns expected for lactobacilli and thus not considered a probiotic safety concern.

In this study, we have demonstrated in vitro safety data and identified and characterized certain probiotic properties of this human microbiota commensal, as well as properties unique to this strain. For instance, this strain appears to exert broad-spectrum antimicrobial effect. Furthermore, we have begun to define the novel concept of the enhancement of certain probiotic properties through the use of a preformed *LfQi6* biofilm cell mass. On a broader level, we suggest that there may be value in the use of preformed commensal biofilms in the practice of clinically relevant probiotic supplementation, on a data-driven, strain-specific basis. To our knowledge, current commercial probiotic strains worldwide are fermented and harvested using standard planktonic growth conditions. Engraftment of these strains relies on such planktonic microbiota attaching to a human body surface to become resident microbiota. The data contained here indicates that administering probiotics as preformed biofilms may improve the probability of tissue adhesion and thus persistence while potentiating probiotic benefit to the host.

There is limited data directly comparing the probiotic effects of lactobacillus biofilms versus planktonic lactobacilli. To our knowledge, this is the first report comparing the probiotic characteristics of *Lact. fermentum* biofilm cell mass against planktonic cell mass. Data contained in this report not only support the safety of *LfQi6* as a probiotic but also support increased probiotic efficacy for characteristics such as antioxidant benefit and probiotic engraftment, with potentially greater ultimate host benefit when *Lact. fermentum LfQi6* is delivered as a preformed biofilm cell mass as opposed to a standard planktonic preparation. This report suggests the utility of this particular *Lact. fermentum LfQi6* biofilm cell mass and potentially other preformed commensal biofilms in the growing field of probiotic consumer products and microbiome

therapeutics. While we did not address mechanistic interactions in this study, further studies are underway to evaluate and define specific mechanisms of biofilm-unique bacterial-host interactions for the commensal discussed in this study and other human microbiota commensals.

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Compliance with Ethical Standards

Conflict of Interest Drs. Berkes and Monsul report the following disclosures: Co-founders of Quorum Innovations, LLC (Sarasota, FL). They are also co-inventors on several patents in the area of the human microbiome and biofilm modulation. The remaining authors are or were affiliated with Quorum Innovations, LLC.

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