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Select Probiotics Exhibit Antioxidative and Anti-Inflammatory Properties for Gut Modulation: *In Vitro* Analysis

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Abstract

The gut microbiota harbors a complex ecosystem of bacteria that govern host health homeostasis. Alterations to the intestinal environment, known as gut dysbiosis, is associated with several diseases. Targeting the gut with microbiome-engineered therapeutics, such as probiotics, is a promising approach to restore microbial homeostasis and host health. Probiotics can effectively improve the gut environment, although strain-specific mechanisms remain largely unknown. Thus, this study aims to identify beneficial action of select probiotics to modulate the gut environment through antioxidative and anti-inflammatory properties. To this end, we tested the interaction among three probiotic strains – *Lactobacillus gasseri* A237 (LgA237), *Lactobacillus plantarum* WCFS1 (LpWCFS1) and *Lactobacillus fermentum* NCIMB 5221 (Lf5221) – and a human intestinal epithelial cell line, HT-29, for adhesion properties, radical scavenging abilities and anti-inflammatory activities. All three probiotics adhere well to HT-29 cells, indicating proper gut colonization. LpWCFS1 demonstrated the greatest adhesion capacity (68.3%), followed by LgA237 (35.5%) and Lf5221 (25.9%). The probiotics also exhibit excellent antioxidant properties via DPPH radical scavenging activity, comparable to quercetin, a known and potent antioxidant. Moreover, LgA237, LpWCFS1 and Lf5221 decrease interleukin-8 expression in lipopolysaccharide (LPS)-damaged HT-29 cells (41.19, 34.53 and 14.80% reduction, respectively), compared to non-treated cells. Further investigation of LpWCFS1 and LgA237 revealed a significant ($p<0.0001$) reduction in monocyte chemotactic and activating factor (MCAF) protein expression by 63.81 and 60.33%, respectively, in colitis-induced IECs. Overall, our results indicate adhesion, antioxidative and anti-inflammatory therapeutic potential of the tested probiotics through antioxidative and anti-inflammatory activities. These findings may be used to further understand the role of the tested probiotics in treating inflammation that underlies gut-related diseases. Such knowledge is essential for the development and translation of novel, targeted probiotic therapies to beneficially modulate the gut environment and reduce inflammation, improving host health.

Keywords: Gut microbiome, probiotics, therapeutics, inflammation, oxidative stress

Introduction

The human gastrointestinal (GI) tract harbors a complex microbial ecosystem made up of trillions of microorganisms, bacteria and fungi, known as the gut microbiome[1]. The gut microbiota is considered as the body's "essential organ"[2], as it regulates nutrient metabolism, immune signaling, pathogenic threats and integrity of the GI tract, playing an imperative role in host health[3]. Various lifestyle and environmental factors can cause shifts in the gut bacterial community, resulting in microbial dysbiosis[1]. Dysbiosis can promote a chronic inflammatory state in the gut, increasing intestinal permeability and impairing metabolic processes within the gut. The systemic consequences of gut dysbiosis include chronic, low-grade inflammation and the development or progression of diseases, such as obesity, type 2 diabetes, unhealthy aging and inflammatory dermatoses[4]. Systemic chronic inflammation alters immune regulation and tissue or organ function, increasing the risk for several diseases across the lifespan[5]. Many signaling pathways are involved in systemic inflammation, posing significant therapeutic challenges. Due to its role in immune response modulation, the gut microbiome is an opportunistic target for mitigating systemic inflammation.

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Targeting the gut using probiotic formulations to manipulate the microbiome is an attractive option to restore microbial homeostasis and host health[6]. Probiotics are live microorganisms that elicit health benefits to the host when administered in adequate amounts[7]. Probiotics are available in the form of functional food products (e.g., yogurt, cheese, kefir), as well as dietary supplements. The efficacy of probiotics occurs through modulation of the gut microbial composition and function. Probiotics can mitigate gut dysbiosis by increasing the abundance of beneficial genera, such as *Lactobacilli* and *Bifidobacteria*, while inhibiting the growth of harmful species[8]. Moreover, probiotics produce metabolites that support the biotransformation of nutrients present in the intestine[9]. Most notably, probiotics within the *Lactobacillus* and *Bifidobacterium* genera ferment non-digestible carbohydrates to produce lactic acid, while many also form short-chain fatty acids (SCFAs), bile-salt hydrolase, ferulic acid and other beneficial enzymes. As a result, vitamin synthesis occurs, alongside free radical scavenging, antioxidant and anti-inflammatory activities[10], thereby stimulating an immune response[11]. Probiotics and their metabolites may act on the immune and non-immune cells (e.g., intestinal epithelial cells; IECs) to downregulate pro-inflammatory cytokines (e.g., interleukin (IL)-8)[12]. However, the metabolic activity of probiotics are often strain- or species-specific.

The *L. plantarum* WCFS1 (LpWCFS1) probiotic strain exerts bile salt hydrolase (BSH) activity and produces anti-inflammatory SCFAs[13]. Microbe-derived BSH deconjugates bile salts, which are detrimental to the intestinal barrier and trigger inflammation. The parental strain of LpWCFS1, *L. plantarum* NCIMB 8826, improves metabolic markers in diabetic and obese *Drosophila* models[4], gut modulation in metabolic syndrome hamster models[14], and colon and skin inflammation in hyperlipidemic human *APOC1*(+/+) transgenic mice[15]. *L. fermentum* NCIMB 5221 (Lf5221) is another beneficial probiotic, recognized for its production of ferulic acid esterase (FAE), an enzyme that enables GI microbes to form ferulic acid, a potent phytochemical with anti-inflammatory and antioxidative properties[16]. The FAE-activity of Lf5221 facilitates the reduction of metabolic disease markers, as evident in previous *in vivo* studies[4, 17]. Finally, *Lactobacillus gasseri* is a promising probiotic for therapeutic action against gut inflammation and metabolic dysfunction. Twelve-week supplementation of *Lactobacillus gasseri* BNR17 in mice and humans reduces body weight and visceral fat, respectively[18]. Similarly, *L. gasseri* SBT2055 prevents HFD-induced body weight, visceral fat mass and elevated intestinal permeability in mice[19]. The beneficial activity of *L. gasseri* may be due to antioxidative and anti-inflammatory action, as evident in colitis murine models[20].

The supporting evidence implies therapeutic potential of *L. plantarum, L. fermentum* and *L. gasseri* species for gut-related inflammation and metabolic distress. The present study aimed to explore antioxidative and anti-inflammatory properties of specific strains within the discussed species, namely LpWCFS1, Lf5221 and *L. gasseri* A237 (LgA237). These specific *Lactobacilli* strains harbor unique metabolic activity, according to the above-mentioned reports, that may benefit the gut environment. However, direct investigation of their effects on human gut models is warranted.

Materials-Methods

Materials and Reagents

Probiotic strains LpWCFS1 and LgA237 were obtained from Biena Inc. (Saint-Hyacinthe, QC, Canada). Lf5221 was revived from frozen stock (-80°C). MRS powder was purchased from Millipore Sigma (CAT# 69966; Massachussetts, USA). Human colorectal adenocarcinoma (HT-29) cells were generously provided by Professor Syaram Pandey in the Department of Chemistry and Biochemistry (University of Windsor). Other chemicals used include Dulbecco's Modified Eagle's Medium (DMEM; CAT# D6429; Sigma Aldrich, United States), Trypsin-EDTA (CAT# T4049, Millipore Sigma, Canada) and Fetal Bovine Serum (FBS; CAT# FB12999102), Penicillin-Streptomycin-Glutamine 100X (PSG; CAT# 10378016) and PBS (CAT# 10010049) from Thermo Fisher Scientific (Canada). For the assays, ELISA interleukin (IL)-8 was purchased (CAT# EKC40086-96T; Biomatik, Ontario, Canada), as well as Multiplex ELISA (CAT# 10001, Anogen, Canada) and DPPH (CAT# 300267-50MG, Sigma Aldrich, Canada). Lipopolysaccharide (LPS) from *E. coli* O55:B5 (LPS; CAT# L2880; Millipore Sigma, USA), human interferon gamma (IFN-γ; CAT# ab9659; Abcam, UK), tumor necrosis factor (TNF-α; CAT# 300-01A; Thermo Fisher Scientific, Canada) and IL-1α (CAT# 200-01A; Thermo Fisher Scientific, Canada) were also purchased.

Preparation of Bacterial Strains and Culture Conditions

Probiotic strains LpWCFS1, LgA237 and Lf5221 were grown overnight in MRS at 37°C and resuspended to the desired concentrations. To prepare the cell-free supernatant (CFS), the bacterial cultures were centrifuged at 4000 rpm for 30 minutes and the supernatant was filter-sterilized using a 0.22 µm filter and stored in -20°C until use.

Mammalian Cell Culture

HT-29 cells were cultured in DMEM supplemented with 10% FBS and 1% PSG. Cells from passages 28-40 were used and grown at 37°C in a 5% CO_2 atmosphere. Media was changed every 2-3 days until cells reached confluency.

Bacterial Adhesion

To investigate adhesive properties of the probiotics, HT-29 cells were seeded in a 24-well plate ($5x10^5$ cells/well) in DMEM (10% FBS) and grown to a confluent monolayer. The individual probiotics were resuspended to $5x10^6$ CFU/mL for an MOI of 10:1 (probiotics: epithelial cells). HT-29 cells were washed with PBS and treated with 1mL of the probiotic suspension, or DMEM as control. The infected plate was incubated for 2

hours at 37°C. After removing the well contents and washing the wells, the adhered cells were trypsinized. DMEM was added to each well and ten-fold dilutions were plated on MRS to perform colony counts (CFU/mL) of the adhered bacteria. Percent adhered was calculated using the following equation:

$$\% \text{ Adhered} = \frac{\text{\# of adhered bacteria}}{\text{\# of added bacteria}} \times 100\%$$

Antioxidant Assay via DPPH Radical Scavenging Activity

The probiotics' CFS were evaluated for total antioxidant activity of 2,2-diphenyl-1-picryl-hydrazyl (DPPH), modified from Izzudin et al.[21]. Briefly, 0.2mM methanolic DPPH solution and the CFS' were incubated at room temperature in the dark for 30 minutes. Quercetin (5.5µM solution) and DPPH solution were used as the positive and negative controls, respectively. Absorbance was measured at 517nm, with duplicate readings for each sample. The relative concentration of DPPH (µg/mL) was quantified using a standard curve of DPPH. DPPH radical scavenging activity was calculated using the following equation:

% DPPH radical scavenging activity= [(Abs.of control-Abs.of sample)/(Abs.of control)] X 100

LPS-Induced Disruption and Interleukin-8 Measurement

To evaluate anti-inflammatory metabolic activity of the probiotics, IL-8 expression in IECs was measured. HT-29 cells were plated on a 96-well plate ($2X10^4$ cells/well) until confluent. To stimulate IL-8 production, cells were pre-treated with interferon-gamma (IFN-γ; 100 ng/mL) for 12 hours, followed by LPS (100 ng/mL) with probiotic CFS (10% *v/v*), or PBS (10% *v/v*) as control, for 24 hours. An ELISA assay was performed using the cell culture supernatant, according to the manufacturer's instructions. Each group contained n=5 independent samples.

Colitis-Induction and Pro-Inflammatory Analysis

To investigate the therapeutic potential of the probiotics' CFS on intestinal inflammation, a colitis *in vitro* model was established using IECs. Adapted from Mohebali et al.[22], HT-29 cells were seeded in 96-well format ($1X10^4$ cells/well). Once confluent, the cells were treated with a cytokine cocktail (100 ng/mL TNF-α, 50 ng/mL IFN-γ and 25 ng/mL IL-1α) and incubated for 12 hours. The cells were then treated with LPS (100 ng/mL) and probiotic CFS (10% *v/v*) for 24 hours. Normal media acted as the control. The expression of pro-inflammatory cytokine monocyte chemotactic and activating factor (MCAF) was measured via ELISA with absorbance readout (450 nm).

Statistical Analysis

All statistical analysis were conducted using [JMP] software. Data is presented as means ± standard error. Significance was assessed with one-way ANOVA. All independent groups were compared using Tukey's post hoc analyses. Statistical significance between groups is marked as *p<0.05, **p<0.01 and ***p<0.0001.

Results

Probiotics *L. gasseri* A237 and *L. plantarum* WCFS1 Demonstrate Good Adherence to Intestinal Epithelial Cells

The probiotics were evaluated for their ability to adhere to IECs. Among the three tested strains, LpWCFS1 showed the greatest adhesion capacity, with an average of $3.41X10^6$ CFU/mL adhered (68.3% adhered; Figure 1). Adhesion of LpWCFS1 was significant compared to control (p<0.0001), LgA237 and Lf5221 (p=0.0072 and p=0.0015, respectively). Notably, the adherence of LpWCFS1 was 92.7% higher than LgA237 adhesion, while it exceeded Lf5221 adhesion by more than two-fold. Although less than LpWCFS1, LgA237 and Lf5221 also demonstrated good adhesion to HT-29 cells. Incubation with LgA237 led to significant adherence of $1.77X10^6$ CFU/mL of live bacteria (35.5% adhered; p=0.0045), compared to control. Although treatment with Lf5221 showed the least adherence, a significant effect was observed with $1.29X10^6$ CFU/mL adhered (25.9% adhesion; p=0.02). Overall, individual exposure to the three tested probiotics enabled adhesion of live bacterial cells to IECs.

Probiotics LpWCFS1 and LgA237 Produce Antioxidant Compounds

The CFS from LgA237 and LpWCFS1 were tested for antioxidant properties and compared against quercetin, a well-known antioxidant. Our results show excellent DPPH radical scavenging activity of all three probiotics (Figure 2). A significant reduction in DPPH concentration resulted from treatment with all probiotic CFS and quercetin, compared to control (p<0.0001). The antioxidative effects of LpWCFS1 (96.0%) and LgA237 (97.0%) slightly exceeded that of quercetin (94.6%), although not significantly. Moreover, Lf5221 exhibited equivalent antioxidant properties as quercetin, scavenging 94.6% of DPPH radicals. The untreated sample had 47.5 ± 0.97 µg/mL of DPPH present, while the probiotic- or quercetin-treated groups contained at most 2.6 µg/mL, indicating a minimum 94.5% reduction in DPPH levels. Treatment with LgA237 resulted in the greatest decrease in DPPH concentration (1.5 ± 0.17 µg/mL), followed by LpWCFS1 (2.0 ± 0.02 µg/mL) and finally Lf5221 and quercetin (2.6 ± 0.15 and 2.6 ± 0.36 µg/mL, respectively). In sum, the three tested bacterial strains demonstrate significant antioxidant activity via DPPH radical scavenging.

***L. plantarum* and *L. gasseri* probiotic supernatants attenuate lipopolysaccharide-induced inflammation in intestinal epithelial cells.**

To assess the anti-inflammatory potential of the probiotic supernatants, the expression of pro-inflammatory cytokine IL-8 was measured in LPS-inflamed HT-29 cells. Treatment with CFS from either LgA237 or LpWCFS1 significantly downregulated IL-8 by 34.2% (2617.9 ± 272.3 pg/mL; p=0.0066) and 29.4%

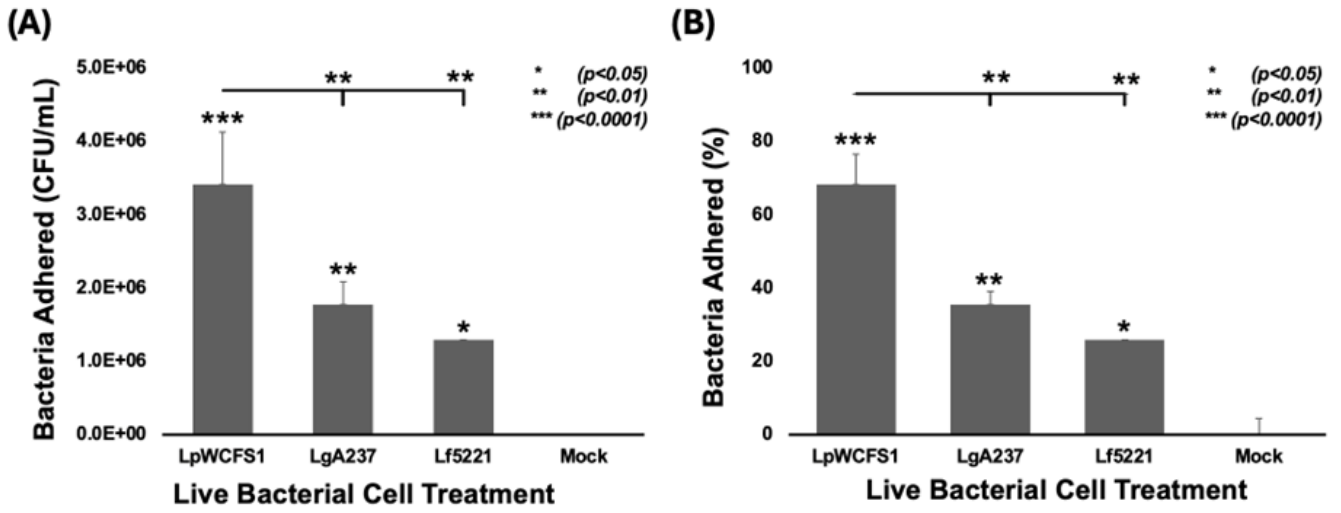

Figure 1. Adhesion of probiotic bacterial cells to HT-29 intestinal epithelial cells represented as (a) CFU/mL and (b) percent adhered. Cell monolayers were treated with live bacterial cells at an MOI of 10:1 (probiotics: epithelial cells) for 2 hours to enable adhesion. The adhered bacteria was isolated and plated on MRS agar for quantification (CFU/mL). Stars indicate significance relative to control (DMEM), bars denote between-group differences and errors bars represent standard error among independent samples (n=3). CFU: colony forming units. Lf5221: *Lactobacillus fermentum* NCIMB 5221. LpWCFS1: *L. plantarum* WCFS1. LgA237: *L. gasseri* A237.

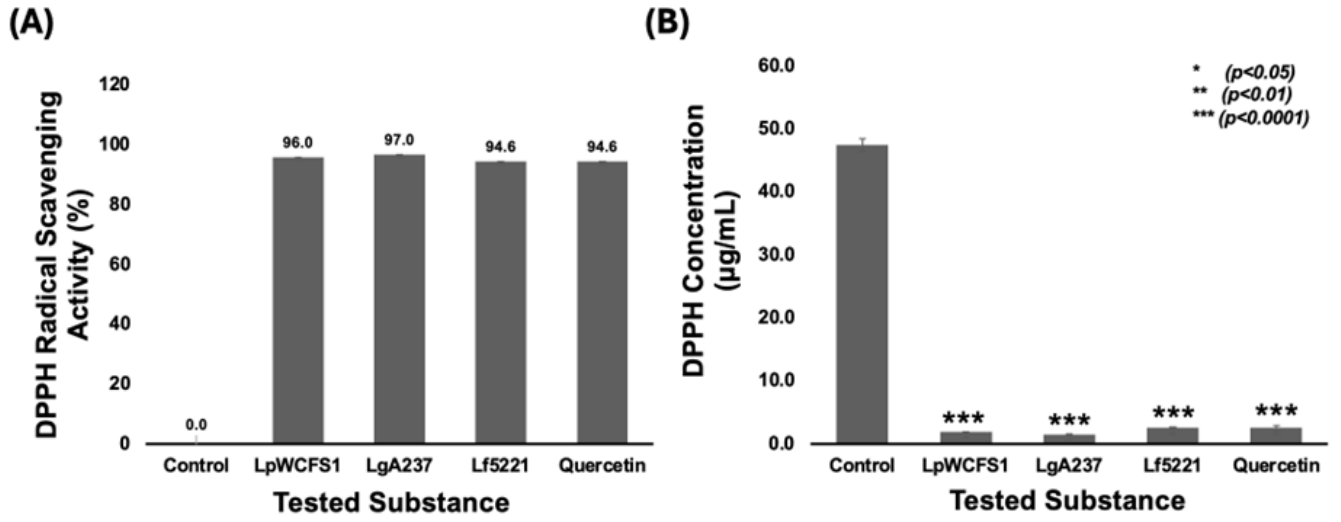

Figure 2. Antioxidant capacity of select probiotics, displayed as (A) DPPH radical scavenging activity and (B) concentration of DPPH (µg/mL). A 0.2mM methanolic DPPH solution was prepared and incubated with either quercetin (5.5µM solution) or probiotic CFS (50% *v/v*) for 30 minutes in room temperature, in the dark. Stars indicate significance relative to control (DPPH solution) and errors bars represent standard error among duplicate readings. LpWCFS1: *Lactobacillus plantarum* WCFS1; LgA237: *L. gasseri* A237; Lf5221: *L. fermentum* NCIMB 5221.

(2805.2 ± 260.2 pg/mL; p<0.0194), respectively, compared to control (3976.1 ± 151.8 pg/mL; Figure 3). Incubation with Lf5221 CFS also led to a decrease in IL-8 expression by 13.8% (3428.14 ± 283.3 pg/mL), compared to control, although not significant. Moreover, there were no differences found between the three probiotic groups. In general, the three tested probiotics showed a significant or promising downregulation of inflammatory IL-8 in inflamed IECs.

Postbiotics from *L. plantarum* and *L. gasseri* Reduce MCAF Inflammatory Protein Expression

To further explore the immunomodulatory effect of LgA237 and LpWCFS1, an ELISA assay was performed to measure monocyte chemotactic and activating factor (MCAF) protein expression. Following colitis induction, treatment with LpWCFS1 or LgA237 significantly reduced MCAF expression by 63.8 and 60.4%, respectively, compared to control (p<0.0001;

Figure 4). Moreover, the MCAF concentration was quantified and revealed untreated cells to have 607.3 ± 46.8 pg/mL. In comparison, cells treated with LpWCFS1 expressed 219.8 ± 13.5 pg/mL of MCAF, while LgA237 treatment showed 240.9 ± 26.6 pg/mL present. Both probiotics significantly downregulated MCAF expression, with no between-group differences.

Discussion

Alterations to the intestinal environment are associated with the development and progression of several diseases, including metabolic disorders. Inflammation in the gut may be triggered by microbial dysbiosis, improper dietary intake and immune dysfunction, among other external factors. The present study aimed to identify anti-inflammatory and anti-oxidative effects

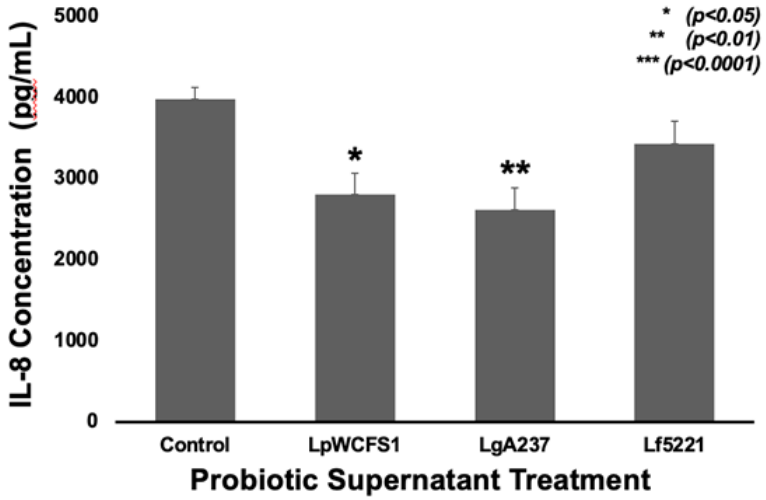

Figure 3. Expression of pro-inflammatory cytokine IL-8 in LPS-stimulated HT-29 cells, with or without probiotic CFS. HT-29 cells were pre-treated with IFN-γ for 12 hours, followed by LPS with or without probiotic CFS (10% *v/v*) for 24 hours. IL-8 expression was quantified compared to a standard curve. Stars indicate significance relative to control (PBS) and errors bars represent standard error among independent samples (n=5). IL-8: interleukin-8; LPS: lipopolysaccharide; CFS: cell-free supernatant; Lf5221: *Lactobacillus fermentum* NCIMB 5221; LpWCFS1: *L. plantarum* WCFS1; LgA237: *L. gasseri* A237.

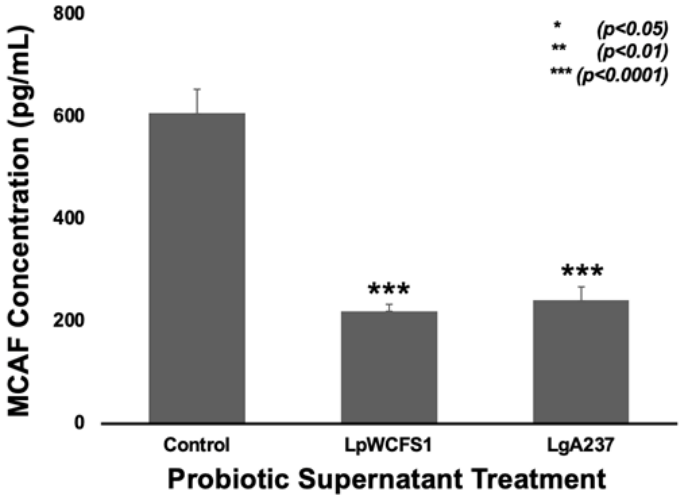

Figure 4. Pro-inflammatory protein expression by colitis-induced HT-29 cells treated with cell-free supernatants from *Lactobacillus plantarum* WCFS1 and *Lactobacillus gasseri* A237. HT-29 cells were treated with a cytokine cocktail to induce a colitis-like phenotype for 12 hours, then treated with LPS (100 ng/mL) with or without probiotic CFS for 24 hours. Stars indicate significance relative to control (PBS) and errors bars represent standard error among the independent samples (n=3). CFS: Cell-free supernatant. LpWCFS1: *Lactobacillus plantarum* WCFS1; LgA237: *L. gasseri* A237; MCAF: monocyte chemotactic and activating factor.

of three *Lactobacilli* strains to benefit the gut environment. Our results demonstrate all three probiotics to reveal favorable colonization and immunomodulatory properties in human IECs.

Probiotic adhesion to intestinal mucosa is important for colonization and protects against enteropathogens through competition for host binding sites[23]. Binding to mucus is an initial step for a probiotic's interaction with the host organism[24]. Upon adhesion, probiotics strengthen IEC tight junctions, inhibiting translocation of harmful gut microbes and metabolites into the bloodstream[25]. Adhesion of live bacterial cells to colon cell monolayers is an established method to evaluate adhesive properties of probiotics. Our findings reveal good adherence of all tested strains to IECs, aligned with earlier research of *Lactobacilli* strain adherence to HT-29 and Caco-2 cells[26]. Various factors contribute to a probiotic's adherence to mucus-producing HT-29 cells, including species-specific mucus-binding proteins, hydrophobicity, steric forces and surface molecules[25, 27]. Although our study did not investigate mechanisms underlying adhesion, previous research has identified a relationship between probiotic surface proteins and adhesion ability. For example, LpWCFS1 expresses mannose-specific adhesin (MSA) which binds to mannose residue in mucin[28]. Similarly, the mucus adhesion-promoting protein (MapA) was shown to mediate the adhesion of *L. fermentum* and *L. plantarum*[24]. S-layer like surface proteins have been found on the surface of various *L. gasseri* strains, which may mediate their adherence to IECs and interaction with immune cells[29]. Other properties such as inhibition of pathogen adhesion, coaggregation and hydrophobicity can be evaluated to further explore the adhesive capacities of the presented probiotics. Overall, all three bacterial strains exhibit good adherence capacity to IECs, suggesting potential to colonize and prolong transit time in the gut for host health benefits.

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense[30]. Excessive ROS production can trigger metabolic dysfunction, induce adipogenesis and promote chronic systemic inflammation, leading to diseases such as diabetes and cardiovascular disease. Supplementation with nutrient antioxidants (i.e., vitamins A, C and E) can strengthen the body's ability to neutralize free radicals. Similarly, supplementation of probiotics may reinforce the antioxidant status, as discussed by a recent meta-analysis[31]. To investigate the potential of the probiotic-produced metabolites to act as free radical scavengers, a DPPH assay was performed. In the presence of antioxidant molecules, DPPH radical solution is reduced and a colorimetric change from violet to pale yellow occurs. This method is widely used to test the antioxidant capacity of molecules from herbal extracts, phenolic compounds or other natural sources[32]. In the current study, the concentration of DPPH significantly decreased in the presence of the probiotics' CFS or quercetin, indicating excellent antioxidant activity by all three probiotics. Quercetin is a potent antioxidant and one of the most well-studied dietary flavonoids present in many vegetables, fruits, tea and red wine[33]. These findings suggest remarkable antioxidative properties of the tested probiotics, as they all exceed or are equivalent to the observed radical scavenging activity of quercetin. The antioxidant activity of LpWCFS1 is likely attributed to its relatively high concentration of intracellular Mn^{2+} ions[34]. A previous study performed by Prete et al.[35] investigated ROS modulation of several *L. plantarum* strains and found LpWCFS1 to exhibit approximately 20.1% DPPH free radical scavenging activity. The authors performed the DPPH assay with slight modifications, including co-incubation of DPPH molecule with live bacterial cells rather than its CFS. Comparatively, our results show substantially higher DPPH radical scavenging activity of LpWCFS1, likely due to more potent antioxidant molecules contained with the probiotic's metabolites rather than the live cells. Due to its production of FAE, Lf5221 was expected to exert antioxidant activity[36]. Our findings confirm the antioxidative effect of Lf5221, aligned with previous studies[37]. Several *L. gasseri* strains have demonstrated antioxidant activity, including *L. gasseri LG*08[38], *L. gasseri SM 05*[39] and *L. gasseri* ATCC 33323[40]. However, to our knowledge, this is the first investigation of the antioxidant activity of *L. gasseri* A237. Additional *in vitro* methods, such as the 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) or ferric reducing antioxidant power (FRAP) assays, can be performed to confirm the antioxidant activity of the tested probiotics' metabolites and live bacterial cells. Further investigation of the probiotics' effects on IEC intracellular ROS production would be beneficial.

Alongside shifting microbial composition, a dysbiotic gut promotes an inflammatory state and disrupts gut function. For example, a dysbiotic gut may increase abundance of Gram-negative peptidoglycan bacteria, such as pathogenic *Escherichia coli*, which contain endotoxin LPS in the outer membrane. LPS impairs IEC lining and increases gut permeability, leading to a "leaky gut" and subsequently entering the bloodstream. Upon translocation, LPS binds to Toll-like receptor 4 (TLR4) and activates a pro-inflammatory signaling cascade via the nuclear factor-κB (NF-κB) pathway[41]. The resulting state of metabolic endotoxemia promotes low-grade, systemic inflammation. IL-8 is a chemokine that is clinically used as a biomarker of systemic inflammation. While IL-8 is not expressed under normal physiological conditions, an upregulation of IL-8 occurs in response to inflammation[42]. In this study, human IECs were exposed to LPS to evoke an inflammatory state that would produce IL-8. The untreated cells expressed a high amount of IL-8 (3976.1 ± 151.8 pg/mL), confirming an inflammatory response. As expected, treatment with either LpWCFS1 or LgA237 CFS significantly decreased the concentration of IL-8, thereby beneficially modulating the immune response. Our findings are aligned with previous research on other *L. gasseri* strains[43]. Similarly, the anti-inflammatory effect of LpWCFS1 was expected based on earlier reporting of LpWCFS1 immunomodulatory benefits[44]. Although treatment with Lf5221 did not result in a significant impact, a downregulation

of IL-8 was observed, indicating promising anti-inflammatory potential. Lf5221 also produces SCFAs[45], which exert anti-inflammatory properties and help maintain proper gut barrier function under an inflammatory state[46]. Thus, our findings for all three probiotics were expected, although additional research on the anti-inflammatory action of Lf5221 would be beneficial.

Following the significant anti-inflammatory action by LpWCFS1 and LgA237, further investigation of the immune response was performed. Alongside IL-8, MCAF plays a pivotal role in inflammatory diseases and is involved in immune signaling pathways, including TLR4[42]. MCAF also contributes to the development of obesity, diabetes, cardiovascular disease and sarcopenic obesity, among other metabolic disorders. Our findings reveal a remarkable reduction of MCAF following treatment with metabolites from LgA237 or LpWCFS1, indicating excellent immunomodulatory action. These findings were expected, given the anti-inflammatory action demonstrated against IL-8 by both bacterial strains. Our results extend earlier reports that state *L. gasseri* Kx110A1 was the only species among several *Lactobacilli* species tested to inhibit cytokine release of LPS-stimulated or pathogen-infected macrophages[47]. Meanwhile, *in vivo* evidence of immune response modulation by LpWCFS1 is well-established[48]. Further investigation of LpWCFS1 and LgA237 live bacterial cells on MCAF regulation of IECs as well as immune cells would be beneficial.

The present study is among the first studies to evaluate specific *Lactobacilli* strains – LpWCFS1, LgA237 and Lf5221 – for their adhesion to HT-29 cells and immunomodulatory effects in LPS-inflamed IECs. To our knowledge, there are no previous reports of LgA237 and its bioactivities. As discussed earlier, specific strains of probiotics offer distinct benefits. This study adds to the current literature of *L. plantarum*, *L. gasseri* and *L. fermentum* species, as well as strain-specific properties. Such knowledge is critical for the translation of probiotic species to novel therapeutics.

Conclusion

The gut microenvironment may benefit from probiotics via compositional or functional changes. However, probiotic benefits are often strain-specific and thus require focused investigation. The current study evaluated three specific *Lactobacilli* strains for beneficial effects on the human gut *in-vitro*, including adhesion, antioxidative and anti-inflammatory properties. The probiotics successfully adhered to human IECs and modulated the immune response following induced inflammation, indicating promising biotherapeutic potential to mitigate gut inflammation. The demonstrated local effects to the gut may have downstream benefits to systemic health, given the impact of the gut microbiota on whole-body health.

This study investigated functional changes at a cellular level, however, future research may explore gut microbial modulation following treatment with these select bacterial strains. Additional mechanistic studies can be performed to determine the targeted immune pathways by the tested probiotics. The use of probiotics as gut therapeutics is an evolving and exciting field of research. Probiotics and their byproducts can be translated as novel therapies for general human health or disease-specific indications. Moreover, probiotics may be genetically engineered to enhance specific properties for desired applications. Probiotic-based drug delivery systems, such as encapsulation, can also be designed to enable targeted delivery of live bacterial cells or their metabolites. However, significant research is needed prior to the translation of live bacterial cells, including additional *in vitro* screening and optimization of strain-specific benefits. Following demonstrated safety and efficacy *in vivo*, clinical trials may be performed in general and diseased human populations. Overall, microbiome-engineered therapeutics hold the potential to push the boundaries of current therapeutics, advancing the fields of biotechnology, engineering and more.

Authors' contributions

Boyajian JL, Abosalha A and Chen J performed the in vitro experiments and analyses. Prakash S supervised the research design and methodology and manuscript preparation. Islam P, Kassab A, Santos M, Shum-Tim C, Renesteen E and Makhlouf S contributed to the interpretation of results and reviewed the manuscript. Boyajian JL wrote the manuscript, with assistance from Chen J.

Availability of data and materials

All relevant data is presented within the manuscript. Additional raw data will be available upon request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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