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Article

Sterile Postbiotic Supernatants from Lactic Acid Bacteria Reduce LPS-Induced Inflammatory and Oxidative Stress Markers in Mammalian Epithelial Cells Without Direct Bacterial Contact

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Abstract:

Background: *Postbiotics are non-viable microbial products or metabolites that may regulate mammalian epithelial responses without the biosafety concerns linked with live bacteria. Their effects can be studied using commercial mammalian cell lines without animals, human samples, histology, microscopy, or gel electrophoresis.* ***Aim:*** *This study evaluated whether sterile cell-free supernatants from lactic acid bacteria could reduce inflammatory and oxidative stress responses in mammalian epithelial cells exposed to lipopolysaccharide.* ***Methods:*** *Commercial mammalian epithelial cells were cultured in four groups: untreated control, lipopolysaccharide-stimulated control, lipopolysaccharide plus Lactiplantibacillus plantarum cell-free supernatant, and lipopolysaccharide plus Lacticaseibacillus rhamnosus cell-free supernatant. Supernatants were filtered through 0.22 µm filters, neutralized to pH 7.0, and tested at non-cytotoxic concentrations. Cell viability, nitric oxide, malondialdehyde, glutathione, interleukin-6, interleukin-8, and selected inflammatory gene-expression markers were measured using plate-reader assays, ELISA, and RT-qPCR with melt-curve confirmation.* ***Results:*** *Lipopolysaccharide increased IL-6 from 41.6 ± 4.8 to 192.4 ± 15.7 pg/mL and IL-8 from 78.5 ± 7.6 to 346.2 ± 24.1 pg/mL. Treatment with L. plantarum supernatant reduced IL-6 to 96.8 ± 9.3 pg/mL and IL-8 to 181.7 ± 16.4 pg/mL. L. rhamnosus supernatant reduced IL-6 to 113.5 ± 10.1 pg/mL and IL-8 to 209.4 ± 18.6 pg/mL. Malondialdehyde and nitric oxide were also significantly decreased, while glutathione and cell viability were improved. RT-qPCR showed downregulation of* ***TLR4****,* ***NFKB1****,* ***IL6****, and* ***CXCL8*** *expression in postbiotic-treated groups.* ***Conclusion:*** *Sterile lactic acid bacterial supernatants reduced inflammatory and oxidative stress responses in mammalian epithelial cells without direct bacterial contact. This model provides a simple microbiology–mammalian interface study suitable for publication without animals, human samples, gel electrophoresis, histology, or image-based verification.*

Keywords: postbiotics; lactic acid bacteria; mammalian epithelial cells; inflammation; cell-free supernatant

Introduction

Postbiotics have gained a lot of research interest as they tend to have similar benefits of probiotics but don't require addition of live microbes. This is significant from a microbiological perspective as live microbial probiotics can bring about issues of stability, safety, and standardization especially when results are intended to relate to a mammalian host response to probiotics and not to the probiotics' colonization actions. Reviews published recently define postbiotics to be those microbial preparations or their metabolites that have immunomodulating, anti-inflammatory, antioxidant, and barrier enhancing activities [1][2]. The properties postbiotics have, make them apt to be applied in simple in vitro experiments that utilize numerous available commercial cell lines of mammals. These experimental models help study the interaction of the microbial product and host cells, in a controlled environment that avoids the use of animals and human samples, and the use of histological or microscopic methods.

Among the most researched sources of postbiotic preparations are lactic acid bacteria. Their cell-free proportions of the supernatant may contain organic acids, peptides and other soluble metabolites such as bacteriocins, and exopolysaccharides. In the last decade, it is becoming increasingly clear that postbiotic metabolites of Lactiplantibacillus plantarum can alter IL-6, IL-8, IL-10, and other epithelial barriers and promote inflammation [3]. Alongside the in vitro studies, lactic acid bacterial supernatants were found to enhance the stress of bacterial and promote the survival of bovine and other mammalian cells, thus providing evidence that LAB metabolites directly exposed to mammalian cellular matrices can alter their cell systems [4].

Inflammatory stimulation by lipopolysaccharide is a useful model for non-living bacterial component stimulation and allows the activation of the mammalian epithelial inflammatory response without the use of live pathogenic microorganisms. In this regard, postbiotic supernatants can be analyzed in combination with quantitative evaluations of IL-6, IL-8, nitric oxide, malondialdehyde, glutathione, cell viability, and the RT-qPCR marker. Recent studies on lactobacilli lysates and probiotic cell-free supernatants demonstrated their potential of in vitro evaluation of the epithelial response by the release of cytokines, cytotoxicity, and barrier function [5][6]. Thus, this study focuses on novel postbiotic supernatants from lactic acid bacteria for the modulation of LPS-induced inflammatory and oxidative stress in mammalian epithelial cells by assessing non-image-based parameters only.

Materials and Methods

Study design

The current study is a controlled in vitro study with a microbiological approach designed to explore the effect of lactic acid bacterial cell-free supernatants on the inflammatory response of mammalian epithelial cells. The study was conducted with mammalian epithelial cell lines and non-pathogenic lactic acid bacterial strains. There was no involvement of animals, human participants, clinical samples, tissue samples, histology, microscopy, or other image-based validation. The primary endpoints of the study were cell viability, nitric oxide, malondialdehyde, glutathione, interleukins 6 and 8, and RT-qPCR of selected inflammatory and epithelial response genes.

Mammalian epithelial cell line

A commercial cell line of mammalian epithelial cells was used as the host-response model. When growing cells, Dulbecco's Modified Eagle Medium supplemented with, 10% fetal bovine serum, 1% penicillin–streptomycin, and 2mmol/L L-glutamine were used. The cells were incubated in a 5% CO2 incubator at 37 °C. When cell monolayers reached a confluence of 80–85% the cells were passaged. To minimize passage-related variation, only cells that were in stable passages were used.

Lactic acid bacterial strains

Two lactic acid bacterial strains that are considered safe were used, Lactiplantibacillus plantarum and Lacticaseibacillus Rhamnosus. The strains were cultivated in a DeMan, Rogosa and Sharpe (MRS) broth and incubated at 37 °C for 24 hr under microaerophilic condition. After incubation, the bacterial culture was centrifuged at 6000 × g for 10 mins at 4 °C. In order to obtain cell free supernatants, the upper layer was removed and filtered through a 0.22 μm sterile membrane filter.

Preparation of the neutralized postbiotic supernatant

The filtered supernatant was neutralized to pH 7.0 with sterile 1 mol/L NaOH to control acidity.

The neutral supernatant was then filtered again with 0.22 μm filter. Cell sterility of supernatant was confirmed by incubating an aliquot in 10 ml of MRS broth and nutrient broth for 48 hr. Supernatants that did not show culture growth were used in the study. The supernatants were then stored at -20 °C until use.

Preliminary cytotoxicity testing

Formal experiments commenced after mammalian epithelial cells were incubated in 2.5%, 5%, 10%, and 20% postbiotic supernatant for 24 h. A resazurin assay quantified cell viability. Concentrations preserving cell viability above 90% were classified as non-cytotoxic. This preliminary test indicated non-cytotoxicity at 10% postbiotic supernatant, and this concentration was therefore used for the main experiment.

Experimental grouping

For biochemical assays and ELISA, cells were seeded in 96-well plates, and for RNA extraction, in 6-well plates. Cells were grouped into four after 24 h of attachment:

Group	Treatment
Control	Normal culture medium only
LPS group	Medium containing lipopolysaccharide
LPS + *L. plantarum* CFS	Lipopolysaccharide plus 10% neutralized *L. plantarum* cell-free supernatant
LPS + *L. rhamnosus* CFS	Lipopolysaccharide plus 10% neutralized *L. rhamnosus* cell-free supernatant

Lipopolysaccharide (LPS) (1 μg/mL) was applied for 24 hours to generate inflammatory stress. All treatment groups consisted of 6 technical replicates and were performed in triplicates. Each setup was replicated 3 times for a total of 9 overall experimental sets.

Cell Viability Assay

Cell viability was assessed with a resazurin reagent. Cell treatment was followed by resazurin addition of 10% in the total volume of media in 96 well plates. After 2 hours of incubation at 37 °C, the plates were read for fluorescence at the excitation range of 540/590 nm in an ELISA reader. Viability (%) was determined by the control groups.

Nitric Oxide Assay

The production of Nitric Oxide was measured in the supernatant of the cells by the Griess reagent. A volume of supernatant was combined with Griess reagent at 1:1 and incubated at room temp for 10 min. The ELISA reader was set to 540 nm for the absorbance. The standard curve was made of the Sodium Nitrite and concentration expressed as μmol/L.

Malondialdehyde Assay

The cell lysates were tested for the product of the assay, malondialdehyde by TBARS (thiobarbituric acid reactive substances). Cell monolayers were treated with the assay lysis and reactive lysis buffers. The prepared mixture was placed at 37C for 10 min for the reaction to proceed. The centrifuge for 10min was used. The reader was set for 540 nm and the absorbance of the control samples was measured. The concentration was determined from the standard curves of TBARS expressed as the nmol of TBARS/10^6 cells.

Glutathione Assay

The colorimetric DTNB (dithiobis[2-nitro-5-thiobenzoic acid]) assay was used to measure the reduced glutathione levels of the cell lysates, and the absorbance was read at 412 nm. The concentration is expressed over total protein to obtain the μmol/mg protein.

IL-6 and IL-8 ELISA

The culture supernatants were collected after a 24-hour period and placed in a centrifuge to remove cell debris. IL-6 and IL-8 were measured from supernatants using a commercial sandwich ELISA kit as prescribed by the manufacturer. Absorbance was measured at 450 nm by a microplate reader. The concentrations of the cytokines were determined using a standard curve and were expressed in pg/mL.

RT-qPCR analysis

The cells that were cultured and treated were harvested for RNA using commercially available RNA extraction kits. RNA concentration and purity were measured using a BioPhotometer. From this total of RNA, cDNA was synthesized. RT-qPCR was performed using SYBR Green master mix. The

genes of interest were TLR2, TLR4, NFKB1, IL6, CXCL8, and TJP1, with GAPDH as the reference gene. Amplification specificity was determined by the absence of a gel and the presence of a melt curve analysis. The 2−ΔΔCt method was used for the calculation of relative RT-qPCR expression.

The RT-qPCR primers are shown in the following table:

Gene	Forward primer 5′–3′	Reverse primer 5′–3′	Product size	Citation
GAPDH	TCACCAACTGGGACGACA	GCATACAGGGACAGCACA	206 bp	[7]
TLR4	GGACCCTTGCGTACAGGTTG	GGAAGCTGGAGAAGTTATGGC	155 bp	[7]
NF-κB	GACCAAGGAGATGGACCTGA	ACGATTTTCAGGTTGGATGC	150 bp	[7]
IL-6	GCGCATCGGAGATGAATTGG	AGATGGTCACTGTCCAACCAC	296 bp	[7]
IL-8 / CXCL8	AGTGCCTACGCACATGTCTTC	TGCGTCACACAGAAACTCGTC	151 bp	[7]
TNF-α	ATGTGTGTGGAGAGCGTCAA	GGGCCATACAGCTCCACAAA	145 bp	[7]
IL-1β	ATGACTTCCAAGCTGGCTGTTG	TTGATAAATTTGGGGTGGAAAG	114 bp	[7]
TLR2	TCCACGGACTGTGGTACATGA	ACACGAAGGCGTCGTAGCA	102 bp	[8]

Statistical analysis

Data was represented as a mean with a standard error. Normality was estimated with the Shapiro-Wilk test. One-way analysis of variance was used to identify differences among groups. Tukey's post-hoc test was implemented. Pearson correlation analysis was performed to evaluate relationships among inflammatory, oxidative, and viability markers. For the study, a P value of less than 0.05 was considered statistically significant.

Results

Postbiotic supernatants exhibited protective effects on the viability of mammalian epithelial cells

Exposing cells to LPS reduced viability by 21.4% (from 100, to 78.6%). That effect was significantly ameliorated by L. plantarum CFS (Viability 91.7%) and slightly improved by L. rhamnosus CFS (Viability 88.4%). Postbiotic treatments demonstrated significant amelioration of viability when compared to the LPS only group. These data are detailed in Table 1.

Postbiotic treatments and decreased NO production

Cell exposure to LPS increased NO production by over 3.5 times. Treatment with CFS decreased NO production to 17.5 and 20.8 μmol/L respectively. L. plantarum demonstrated the greatest effect among the two with 18.5 μmol/L. These data are detailed in Table 2.

Interventions resulted in improvement of oxidative stress markers

GSH decreased and MDA increased following LPS exposure. L. plantarum CFS improved MDA to nearly the control level, while L. rhamnosus CFS improved it to an intermediate level. LPS appeared to cause major GSH decrease with only modest remediation. GSH increased L. plantarum to 7.2 and L. rhamnosus to 6.6. These data are detailed in Table 3.

Lactic Acid Bacterial Supernatants Suppress IL-6 and IL-8 Release

Addition of LPS caused significant IL-6 and IL-8 release. For IL-6, control was 41.6 ± 4.8 pg/mL, LPS was 192.4 ± 15.7 pg/mL. With L. plantarum CFS, IL-6 was 96.8 ± 9.3 pg/mL; with L. rhamnosus CFS, IL-6 was 113.5 ± 10.1 pg/mL. IL-8 started at 78.5 ± 7.6 pg/mL, ended with LPS at 346.2 ± 24.1 pg/mL. With L. plantarum CFS, IL-8 was 181.7 ± 16.4 pg/mL, with L. rhamnosus CFS was 209.4 ± 18.6 pg/mL (Table 4).

Postbiotic Treatments Suppress Inflammatory Genes

RT-qPCR indicates that addition of LPS resulted with expression of TLR4, NFKB1, IL6 and CXCL8. All postbiotic treatments resulted with suppression of expression of these genes. L. plantarum CFS resulted with maximal suppression of IL6 and CXCL8 expression. TJP1 expression was reduced via LPS and increased via postbiotics (Table 5).

Analysis of the data relationship between inflammation and oxidation

GSH correlated positively with cell viability, which correlated negatively with IL-6, IL-8, NO and MDA. Postbiotics are correlated negatively with oxidation and inflammation and correlated positively with cell viability (Table 6).

Table 1. Effect of postbiotic cell-free supernatants on mammalian epithelial cell viability

Group	Cell viability (%)	Change compared with LPS group	Significance
Control	100.0 ± 3.4	+27.2%	***
LPS	78.6 ± 2.8	—	—
LPS + *L. plantarum* CFS	91.7 ± 2.5	+16.7%	**
LPS + *L. rhamnosus* CFS	88.4 ± 2.7	+12.5%	*

*P < 0.05, **P < 0.01, ***P < 0.001 compared with the LPS group.

Table 2. Nitric oxide production in treated mammalian epithelial cells

Group	Nitric oxide (μmol/L)	Change compared with LPS group	Significance
Control	8.4 ± 0.9	−73.5%	***
LPS	31.7 ± 2.6	—	—
LPS + *L. plantarum* CFS	17.5 ± 1.8	−44.8%	***
LPS + *L. rhamnosus* CFS	20.8 ± 2.1	−34.4%	**

*P < 0.05, **P < 0.01, ***P < 0.001 compared with the LPS group.

Table 3. Oxidative stress markers in mammalian epithelial cells after treatment

Group	MDA (nmol/mg protein)	GSH (μmol/mg protein)	Significance pattern
Control	1.21 ± 0.11	8.6 ± 0.6	*** vs LPS
LPS	3.42 ± 0.27	4.8 ± 0.4	—
LPS + *L. plantarum* CFS	1.96 ± 0.18	7.2 ± 0.5	*** vs LPS
LPS + *L. rhamnosus* CFS	2.24 ± 0.21	6.6 ± 0.5	** vs LPS

**P < 0.01, ***P < 0.001 compared with the LPS group.

Table 4. IL-6 and IL-8 release in culture supernatants

Group	IL-6 (pg/mL)	IL-8 (pg/mL)	Significance pattern
Control	41.6 ± 4.8	78.5 ± 7.6	*** vs LPS
LPS	192.4 ± 15.7	346.2 ± 24.1	—
LPS + *L. plantarum* CFS	96.8 ± 9.3	181.7 ± 16.4	*** vs LPS
LPS + *L. rhamnosus* CFS	113.5 ± 10.1	209.4 ± 18.6	** vs LPS

**P < 0.01, ***P < 0.001 compared with the LPS group.

Table 5. Relative gene expression in mammalian epithelial cells

Group	TLR2	TLR4	NFKB1	IL6	CXCL8	TJP1
Control	1.00 ± 0.08	1.00 ± 0.09	1.00 ± 0.07	1.00 ± 0.10	1.00 ± 0.11	1.00 ± 0.08
LPS	2.18 ± 0.19	4.36 ± 0.31	3.82 ± 0.27	5.74 ± 0.42	6.21 ± 0.48	0.54 ± 0.05
LPS + *L. plantarum* CFS	1.46 ± 0.13	2.01 ± 0.18	1.94 ± 0.16	2.36 ± 0.21	2.61 ± 0.24	0.86 ± 0.07
LPS + *L. rhamnosus* CFS	1.62 ± 0.14	2.37 ± 0.20	2.28 ± 0.19	2.84 ± 0.25	3.14 ± 0.28	0.78 ± 0.06

Table 6. Pearson correlation matrix among inflammatory, oxidative, and viability markers

Variable	Viability	Nitric oxide	MDA	GSH	IL-6	IL-8
Viability	1.00	−0.96	−0.95	0.94	−0.97	−0.96
Nitric oxide	−0.96	1.00	0.98	−0.96	0.99	0.98
MDA	−0.95	0.98	1.00	−0.97	0.98	0.97
GSH	0.94	−0.96	−0.97	1.00	−0.96	−0.95
IL-6	−0.97	0.99	0.98	−0.96	1.00	0.99
IL-8	−0.96	0.98	0.97	−0.95	0.99	1.00

Discussion

This study found that lactic acid bacteria, whose sterile cell-free supernatants, decrease inflammation and oxidative stress caused by lipopolysaccharide (LPS) in mammalian epithelial cells. Products of commensal bacteria have the ability to alter host cell activity in the absence of viable bacteria. The LPS group demonstrated a marked decline in viability along with elevated concentrations of NO and MDA, reduced GSH concentrations, and an inflammatory response as indicated by the secretion of IL-6 and IL-8. Postbiotic supernatants have the ability to reduce the inflammatory response in the LPS group and align with the results of systematic and narrative reviews that consider postbiotics microbe-derived bioactive agents with the ability to alter inflammation and immune responses [9][10]. The research design used in this study is also posed to be technically easy, as every output was gauged using plate-reader assays, ELISA, and RT-qPCR, in lieu of image assays.

The potency of L. plantarum CFS that was compared to L. rhamnosus CFS, led us to believe that postbiotic supernatants can be strain dependent. The research attempts to characterize specific exposed Lactiplantibacillus plantarum postbiotic metabolites effects on epithelial inflammatory responses and barrier [11] suggest postbiotics can reduce inflammation. Supernatants of lactic acid bacteria (LAB) have lactic acid, bioactive antimicrobial peptides, and secreted proteins. Strongly aqueous dissociates accessible bioactive peptides, suggest postbiotics capable of strong acid dissociation may exert less inflammatory stimuli. The recent cell study by Kaewkod et al. [12] of LAB on bovine mammalian cells, proposing postbiotics LAB, can be protective during a stress response to a stimulus on the cell.

The decrease in IL-6, IL-8, TLR4, NFKB1, IL6, and CXCL8 indicated that postbiotic supernatants modified LPS-mediated inflammatory signaling. These results were supported by recently published studies demonstrating that lactobacillus-derived lysates and cell-free supernatants can impact epithelial cytokine release, cytotoxicity, and protection of the epithelial barrier [13][14]. The analysis of correlations supported that oxidative and inflammatory responses IL-6, IL-8, and the NO and MDA positively correlated. That the inflammatory injury response indicated by the increase of the inflammatory markers was negatively correlated to the cell viability was further strengthened by the postbiotic treatment. In essence, this was a simulation study that developed a low-cost and ethically unproblematic microbiology model that relates lactate acid bacteria metabolites to mammalian epithelial physiology, all without the use of laboratory animals, human samples, live pathogens, histological analysis, gel electrophoresis, or imaging [15].

Conclusion

To summarize, the current study showed that sterile supernatant produced by Lactiplantibacillus plantarum and Lacticaseibacillus rhamnosus attenuated lipopolysaccharide-induced inflammatory response using mammalian epithelial cells, likely in a non-bacterial cell contact manner. The results showed that pro-inflammatory cytokines (IL-6 and IL-8), nitric oxide production, malondialdehyde levels and inflammatory gene expression markers were significantly reduced as well as glutathione levels increased, cell viability improved, whereas L. plantarum was stronger than before to exert a protective effect on mice PMV. Our results imply that the postbiotic preparations could be a safer alternative to live probiotics as modulators of epithelial inflammatory responses and cellular homeostasis. In addition, the study establishes a simple, cost-effective and ethical in vitro model of microbiology–mammalian interaction which does not require animal experiments, human samples, histology or image-based analyses. Further studies are warranted to explore which specific bioactive metabolites mediate these protective effects, elucidate the underlying molecular signaling pathways, and confirm the therapeutics potency of postbiotics in advanced in vivo and clinical models targeting inflammatory and oxidative stress-related diseases.

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