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Posted Date: 3 March 2026

doi: 10.20944/preprints202603.0158.v1

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Article

New Postbiotic Deriving from Sequential Fermentation of Two *Lactocaseibacillus* Strains Exerts Beneficial Effects on Epithelial Gut Barrier and Innate Immunity in Human Enterocytes

Franca Oglio ^{1,2,3,4,*}, Alessia Cadavere ^{1,2,3,4}, Monia De Aloe ^{1,2,3,4}, Anna Lintura ^{1,2,3,4}, Marco Michelini ^{1,2,3,4}, Chiara Luongo ^{1,2,3,4}, Serena Coppola ^{1,2,3,4}, Alessandra Agizza ^{1,2,3,4}, Erika Caldaria ^{1,2,3,4} and Laura Carucci ^{1,2,3,4}

¹ Department of Translational Medical Science, University Federico II, Via Sergio Pansini 5 80131, Naples, Italy

² ImmunoNutritionLab at CEINGE Research Center

³ NutriTechLab Academic Spin-Off

⁴ Task Force for Microbiome Studies, University Federico II, Via Sergio Pansini 5 80131, Naples, Italy

* Correspondence: franca.oglio@unina.it

Abstract

The efficacy of postbiotics vary significantly comparing different strains and preparation processes. We aimed at evaluating the effect of an innovative postbiotic (iPB) generated through the sequential fermentation of *Lactocaseibacillus rhamnosus* GG and *Lactocaseibacillus paracasei* NPB-01, compared to single-strain postbiotics, on epithelial barrier integrity and innate immunity in human enterocytes. Comparative evaluation of the effects elicited by iPB or by single-strain postbiotics on gut epithelial barrier using a Caco-2 cells-based experimental model by measuring cell growth proliferation, tight junction proteins (occludin and zonula occludens 1, ZO-1), mucus protein Mucin-2 (Muc-2), and lactase expression. The modulatory action on the production of innate immunity peptide Human Beta-Defensin 2 (HBD-2) via RT-qPCR and ELISA was also comparatively assessed. iPB exposure resulted in a higher up-regulation of occludin, ZO-1, MUC2 and lactase expression, if compared with the single-strain postbiotics, suggesting a beneficial synergistic action in modulating epithelial gut barrier. Furthermore, iPB induced a significantly higher production of HBD-2, suggesting a synergistic enhancement of innate immune response. Our findings suggested that the sequential fermentation process acts as a biotechnological catalyst, optimizing the immunomodulatory action and gut barrier-protective properties of *Lactocaseibacillus* strains. This study introduces iPB as a high-performance postbiotic candidate for the prevention and management of conditions characterized by alterations of epithelial gut barrier and innate immunity.

Keywords: postbiotic; food allergy; innate immunity

1. Introduction

Postbiotics, which comprise inanimate microorganisms and/or their cellular components and metabolites, are gaining increased attention for their potential health benefits [1]. Although these beneficial actions resemble those of probiotics, the stability and safety of postbiotics make them an appealing alternative in particular for vulnerable populations, including newborns, elderly people, and patients with clinical states for whom the use of probiotics may be not recommended [2–4]. Emerging findings suggest that the health benefits of selected postbiotics can match, and may even exceed those reported for the probiotic counterparts [2,5,6]. Extensive research has uncovered that selected postbiotic products can exert beneficial modulatory actions on microbiome, epithelial barrier, nervous system, immune and metabolic pathways [5,7,8].

Processes commonly used for producing postbiotics include fermentation and microbial inactivation. The fermentation process could increase the microorganisms' population and the production of several cellular components and metabolites, all of which can exhibit functional activities [3]. Microbial inactivation can be achieved through various methods, including heat treatment, sonication, high-pressure, and enzymatic treatment [9]. The effectiveness of postbiotics can vary significantly comparing different strains and preparation processes, thus testing the efficacy of each single postbiotic product is mandatory to ensure it can deliver the intended health benefits [10]. The majority of available data derives from studies on single bacterial strain, but it has been suggested that the presence of multiple microorganisms can increase the beneficial health effects compared to single-strain preparations thanks to the synergistic action of different microorganisms [11,12]. However, most of the products are obtained by growing the microorganisms separately and then combining them in the formulation of the finished product or by co-fermenting two or more microorganisms simultaneously [12,13]. Co-fermentation is based on the simultaneous processing of different living microorganisms to take advantage of the metabolic cooperation, which can lead to improved fermentation performances or the development of products with increased beneficial potential. The dark side of this process could be the possible competition between microorganisms [13].

An innovative postbiotic product deriving from the sequential fermentation of two *Lactobacillus* strains, the *L. rhamnosus* GG and *L. paracasei* NPB-01 has of increasingly developed with the aim to increase the beneficial modulation of body functions. Here we comparatively evaluated the beneficial actions exerted by this innovative postbiotic product vs the two same single heat-inactivated probiotic strains on epithelial barrier and innate immunity in human enterocytes.

2. Materials and Methods

Postbiotic Products

Three different postbiotic products were tested in the study. The first one was obtained from the fermentation of *L. rhamnosus* GG (ATCC 53103) on MRS broth (24 h, 37°C). At the end of the fermentation process, a mild heat treatment was conducted (90°C, 1 min) to inactivate the bacterial biomass, yielding the postbiotic preparation (LGGp). The second one was obtained from the fermentation of *L. paracasei* NPB-01 (DSM 34367) on MRS broth (24 h, 37°C). At the end of the fermentation process, a mild heat treatment (90°C, 1 min) was applied to inactivate the bacterial biomass, obtaining the postbiotic preparation (Lpp). The third one was obtained from the sequential fermentation process using *L. rhamnosus* GG and *L. paracasei* NPB-01 (iPB). Briefly, after the first fermentation of LGG, a mild heat treatment (90°C, 1 min) was applied for inactivating the microbial population. Then, a second fermentation was started by inoculating *L. paracasei* NPB-01. Following the second fermentation, a mild heat treatment (90°C, 1 min) was performed. Both fermentations were carried out on MRS broth for 24h at 37°C. All postbiotics preparation (LGGp, Lpp and iPB) used in this study were dried. All postbiotic products utilized in the study were provided as food grade powder by Science Power Srl (Milan, Italy).

Human Enterocytes Cell Line

For all experiments we used Caco-2 cell line (American Type Culture Collection, Middlesex, UK; accession number: HTB-37). Cells were grown in Dulbecco's modified Eagle's medium with a high glucose concentration (4.5 g/L) (Gibco, Berlin, Germany). The medium was supplemented with 10% fetal bovine serum, 1% l-glutamine, 1% non-essential amino acids, and 1% penicillin/ streptomycin (Thermo Fisher Scientific). Cells were incubated at 37 °C and 5% CO₂. The medium was changed every 2 days.

Postbiotic Stimulation Protocol

Caco-2 cells were seeded (0.1x10⁶ cells/well) in 12-well plates (Corning). After 15 days post-confluence cells were stimulated with the postbiotic products. Caco-2 cells stimulated with medium alone were used as controls. Experiments were carried out in triplicate.

Modulation of Epithelial Gut Barrier

The potential modulatory action on epithelial barrier elicited by the study products was investigated using different tools, as previously adopted [14,15]. Enterocytes cell growth and differentiation were assessed using proliferation assay and brush border enzyme lactase gene expression, respectively. The modulatory action exerted on mucous thickness was explored evaluating mucin 2 (Muc2) gene expression. The effect on tight junctions' network was explored by analyzing the gene expression of two major proteins, occludin and zonula occludens 1 (ZO-1). Lastly, the potential modulatory action on innate immunity was explored analyzing the production of the major innate immunity peptide, Human Beta Defensin (HBD-2), by enterocytes.

Cell Proliferation Assay

Human enterocyte proliferation assays were performed using MTT (the bromide salt of 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium) (Sigma-Aldrich, Milan, Italy). Cells (104 cells/well) were seeded in 24-well plates (Corning, Inc., New York, NY, USA) with or without the postbiotic products at 37 °C in a humidified atmosphere containing 5% CO₂. The cell viability was monitored by adding 5 mg/ml of MTT solution followed by 1 h incubation. The medium was then removed, and the converted dye was solubilized with acidic isopropanol (0.04-0.1 N HCl in absolute isopropanol). Absorbance was read at 570 nm using an Epoch Microplate Spectrophotometer (Bioteck, Winooski, VT, USA).

Real Time PCR

Total RNA from Caco-2 cells was extracted using TRIzol reagent (Gibco BRL, Paisley, UK). The reverse transcription to cDNA was performed with the High-Capacity RNA-to-cDNA™ Kit (Life Technologies, Waltham, MA, USA) following the manufacturer's instructions. The cDNAs were stored at -80°C until analysis. Quantitative real-time PCR (qRT-PCR) analysis, carried out with the TaqMan Gene Expression Master Mix (Applied Biosystems, Vilnius, Lithuania), was used to evaluate the gene expression occludin (Hs05465837_g1) and ZO-1 (Hs01551871_m1). Gene expression of the mucin 2 (Muc2), and lactase enzyme, were evaluated using a SYBR green Master Mix (Applied Biosystems, Grand Island, NY, USA). The primers used for Muc2 were forward (5'-CTCCGCATGAGTGTGAGT- 3') and reverse (5'- TAGCAGCCACACTTGTCTG - 3'). The primers used for lactase were forward (5'-ACACGGTCGATTTCCTCTCT - 3') and reverse (5'-TGGGTTCTTCATGGTGGAGG - 3'). The amplification protocol was 40 cycles of 15 s of denaturation at 95°C, 60 s of annealing at 60°C, and 60 s of elongation at 60°C in a Light Cycler 7900HT (Applied Biosystems, Grand Island, NY, USA). Data were analyzed using the comparative threshold cycle method. We used the glucuronidase beta (GUS-B) gene to normalize the level of mRNA expression (TaqMan probes: Hs00939627_m1; SYBR green Forward primer: 5'-GAAATATGTGGTTGGAGAGCTCATT-3'; SYBR green Reverse primer: 5'-CCGAGTGAAGATCCCCCTTTTA - 3').

Modulation of Innate Immunity

After stimulation with the postbiotics, cell supernatants were collected and stored at -80°C until analysis. Production of HBD-2 was determined by ELISA test, using commercial enzymatic kits (Human DEFβ2/DEFB2, Elabscience Biotechnology Inc. Wuhan, Hubei. Detection limit of 62.5 pg/mL).

Statistical Analysis

The Kolmogorov-Smirnov test was used to determine whether variables were normally distributed. Data were analysed using unpaired t-test. The level of significance for all statistical tests was two-sided, $p < 0.05$. All data were collected in a dedicated database and analysed by a statistician using GraphPad Prism 9.3.0 (La Jolla, CA USA).

3. Results

3.1. Modulation of Human Enterocytes Cell Growth and Differentiation

In preliminary dose-response and time course experiments we defined the best experimental conditions for each postbiotic product. As shown in Figure 1 (panels A and B), both products obtained through the single strain fermentation process (Lpp and LGGp) promoted the growth and the differentiation of human enterocytes. No further increase in cell growth and differentiation was observed adding Lpp and LGGp simultaneously at the maximum dose. On the contrary, the modulatory actions elicited by the postbiotic product deriving from sequential fermentation (iPB) were significantly higher for both parameters, if compared with those obtained by the two single strain postbiotic products alone or in combination.

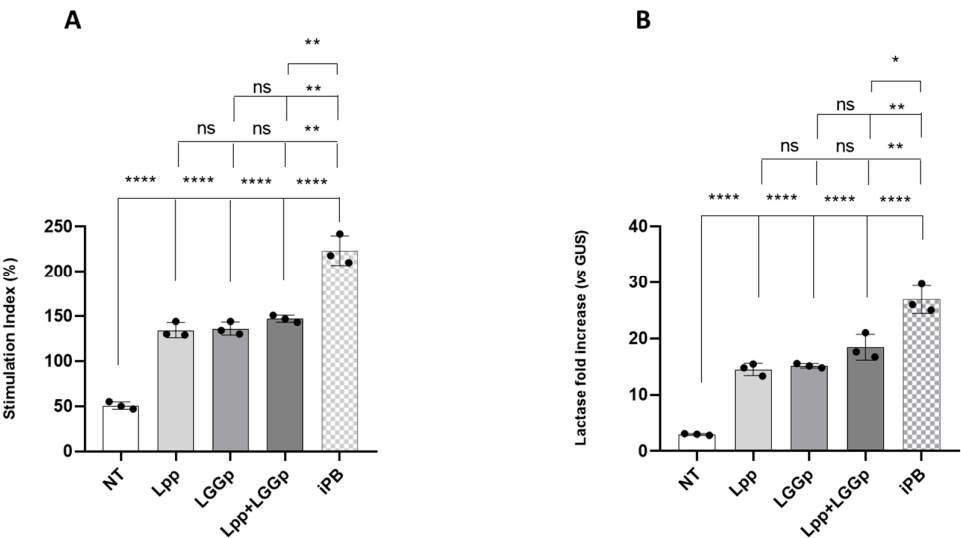


Figure 1. Stimulation index (A) and Lactase fold increase (B) observed on Caco-2 cells stimulated with the postbiotic products. After 15 days post-confluence Caco-2 cells were stimulated for 48 h with 1 mg/ml Lactobacillus paracasei NPB-01 postbiotic (Lpp), with 0.1 mg/ml Lactobacillus rhamnosus GG postbiotic (LGGp), and with 1 mg/ml postbiotic product obtained by sequential fermentation of the two probiotic stains (iPB). The stimulation index was evaluated by MTT test, while the lactase fold increase was evaluated by Real-Time PCR. Data are expressed as mean values and standard deviations of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

3.2. Modulation of Tight Junction Proteins and Mucous Production in Human Enterocytes

As shown in Figure 2, the incubation with all the three postbiotic products led to the increase in the expression of the two major tight junction proteins, occludin and ZO-1 (panels A and B). Also, the expression of the epithelial mucus layer protein MUC2, resulted modulated by these two postbiotic products (panel C). No further stimulatory action for all three parameters was observed adding Lpp and LGGp simultaneously at the maximum respective dose. On the contrary, the iPB consistently induced a significantly greater stimulatory effect on all biomarkers compared to the other postbiotics alone or in combination.

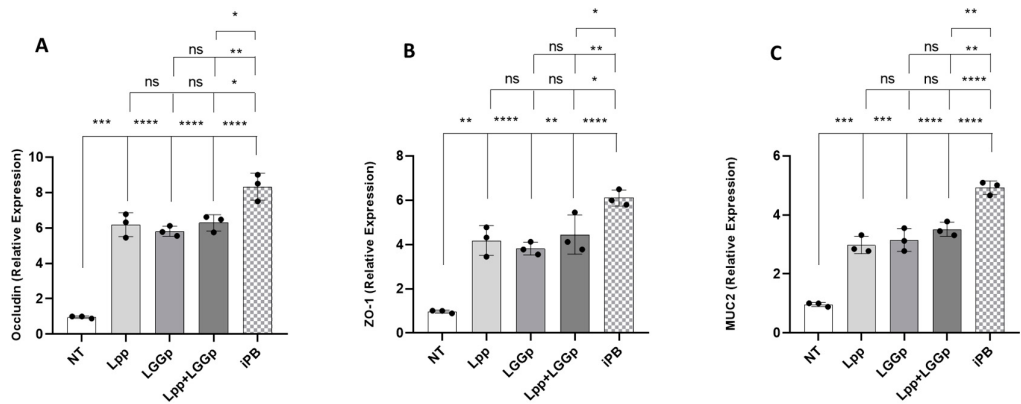


Figure 2. Modulation of the tight junction proteins Occludin (A) and Zonulin-1 (ZO-1) (B), and on mucin2 (MUC2) (C), elicited by postbiotic products. After 15 days post-confluence Caco-2 cells were stimulated for 48h with 1 mg/ml *Lactobacillus paracasei* NPB-01 postbiotic (Lpp), with 0.1 mg/ml *Lactobacillus rhamnosus* GG postbiotic (LGGp), and with 1 mg/ml postbiotic product obtained by sequential fermentation of the two probiotics (iPB). The concentration of occludin, ZO-1, and MUC2 was evaluated by Real-time PCR. Data are expressed as mean values and standard deviations of three independent experiments. *p<0.05; **p<0.01.

3.3. Modulation of Innate Immunity

To investigate the potential modulatory action elicited by the postbiotic products evaluated in this study, we measured the production of the major innate immunity peptide HBD-2 by human enterocytes stimulated with the study products for 48h. As shown in Figure 3, the incubation with all the three postbiotic products led to the increase in the production of HBD-2 by human enterocytes. No further stimulatory action in HBD-2 production was observed stimulating the cells simultaneously with Lpp and LGGp at the maximum respective dose. On the contrary, exposing human enterocytes to iPB resulted in a significantly higher stimulatory effect on HBD-2 production if compared with those observed stimulating the cells with Lpp or with LGGp alone or in combination.

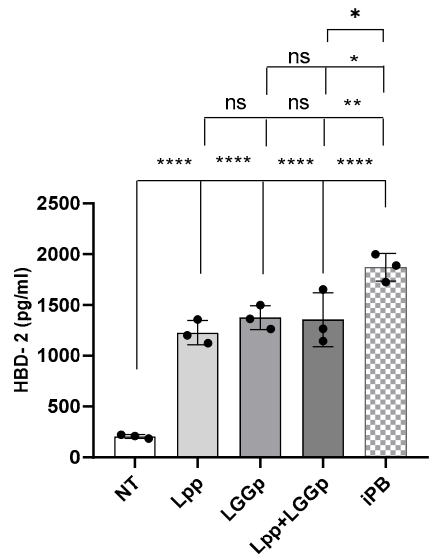


Figure 3. Effect of the postbiotic products on the innate immunity peptide HBD-2. After 15 days post-confluence Caco-2 cells were stimulated for 48h with 1 mg/ml *Lactobacillus paracasei* NPB-01 postbiotic

(Lpp), with 0.1 mg/ml *Lactocaseibacillus rhamnosus* GG postbiotic (LGGp), and with 1 mg/ml postbiotic product obtained by sequential fermentation of the two probiotics (iPB). The concentration of HBD-2, as a marker of innate immunity, was evaluated by immunoenzymatically. Data are expressed as mean values and standard deviations of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

4. Discussion

Fermentation is a primary and essential process for producing postbiotics [16], it could significantly affect composition and function of these products [17–20]. Several studies have highlighted the beneficial effects of different postbiotic products derived from single or multiple bacterial strains, either co-fermented or mixed post-fermentation [21–23]. In this study, we provided the first evidence on the beneficial actions on human enterocytes elicited by an innovative postbiotic (iPB) produced through sequential fermentation of two probiotic strains (i.e., *L. rhamnosus* GG and *L. paracasei* NPB-01). Our findings demonstrated that iPB exerts a more potent regulatory action on human enterocytes proliferation and differentiation, as demonstrated by MTT and brush border enzyme lactase increases, respectively. A parallel more potent effect was also observed on epithelial gut barrier architecture, as suggested by increased expression of the two major tight-junctions' proteins, occludin and ZO-1. We also found that the iPB exposure resulted in a higher increase in the expression of Muc2 and the innate immunity peptide HBD-2 by human enterocytes. Muc2 is a key mucin protein forming the protective mucus layer in the intestine essential for epithelial gut barrier function [24]. Muc2 mucus layer is essential in protecting the host not only against pathogens but also against the occurrence of allergies limiting the exposure to environmental allergens [25]. In addition, stimulating the production of key regulatory factors such as IL-10, Transforming Growth Factor- β (TGF- β), Retinoic Acid (RA), and thymic stromal lymphopoietin (TSLP) by epithelial and dendritic cells (DCs), Muc2 promotes the expansion of Treg cells and reduces inflammation, thereby restoring immune tolerance and homeostasis [25].

Beyond their structural and metabolic roles, intestinal epithelial cells actively participate in mucosal immunity by producing antimicrobial peptides like HBD-2. This small, cationic, antimicrobial peptide "farms" the microbiome, influencing its composition and preventing dysbiosis which can lead to inflammatory conditions. The HBD-2 possesses a broad-spectrum activity against bacteria, viruses, fungi and some parasites. However, in addition HBD-2 activates a variety of immune cells and regulates cytokine/chemokine production, cell migration, proliferation, differentiation, angiogenesis, the wound healing process and maintenance of the epithelial barrier function. Alterations in the level of HBD-2 have been associated with the initiation and development of various inflammatory and allergic diseases [26,27]. Thus, this molecule could be considered as cornerstone of mucosal innate immune system, providing a first line of defence against infections, dysbiosis, inflammation and allergic sensitization [14,28].

For all variables investigated into the study, we found a higher modulatory action elicited by iPB if compared with those obtained by single postbiotics alone or in combination, suggesting that the sequential fermentation process likely induces a unique metabolic signature. We hypothesize that the first fermentation step with LGG modifies the substrate and releases primary metabolites that, following heat inactivation, serve as a specialized matrix for the subsequent fermentation by *L. paracasei* NBP01. This 'metabolic priming' may trigger the synthesis of a more complex array of bioactive compounds, such as specific peptides or metabolites. This study introduces the concept of sequential postbiotic design as a strategy to maximize the health-promoting potential of lactic acid bacteria.

While these results are promising, some limitations should be acknowledged. First, this study focused on two specific probiotic strains, both individually and in a sequential combination; therefore, the reproducibility and generalizability of these effects to other bacterial taxa remain to be established. Additionally, the sequential fermentation was performed exclusively by inoculating LGG followed by *L. paracasei* NBP01. Future research exploring the reversed inoculation order or

different timing intervals could provide deeper insights into how microbial succession shapes the final postbiotic activity.

Furthermore, although the biological impact of iPB is highlighted by the results of this study, the specific effector molecules—whether they be secreted metabolites, cell wall components (such as the capsule), or genomic DNA fragments— as well the respective mechanisms of action, have yet to be fully characterized. It has been suggested that many postbiotics’ effects involve the activation of toll like receptors and related intracellular pathways, such as NF-κB signalling [28]. Thus, the results of this study suggest that the sequential fermentation optimizes the concentration of 'Microbe-Associated Molecular Patterns' (MAMPs) or secreted factors that are recognized by TLRs on human cells [29]. This enhanced modulatory action on epithelial gut barrier and innate immunity suggest that iPB could provide a more efficient protective effect against conditions characterized by alteration of intestinal permeability, dysbiosis, immune tolerance and inflammation. Nevertheless, our findings highlight the potential of sequential fermentation as a novel and superior biotechnological platform for postbiotic production. This approach could pave the way for a new generation of functional ingredients with enhanced efficacy in modulating human health. Further investigations are warranted to test different strain combinations and to broaden the evaluation of intestinal barrier integrity, systemic immunity, and cellular differentiation pathways.

5. Conclusions

In conclusion, our data support the use of sequential fermentation to produce a high-performance postbiotic. iPB stands out as a promising candidate for functional food applications and clinical interventions aimed at strengthening the intestinal barrier and immunity. Future studies involving 'omics' technologies (metabolomics and proteomics) will be essential to pinpoint the exact molecular effectors responsible for these observed synergistic benefits.

Author Contributions: Conceptualization, F.O. and L.C.; methodology, F.O., A.C, M.D.A., A.L.; validation, F.O., S.C. AND L.C.; formal analysis, F.O. AND A.C.; investigation, F.O.; resources, M.M., C.L. AND A.A.; data curation, F.O. AND A.C.; writing—original draft preparation, F.O.; writing—review and editing, L.C.; visualization, F.O.; supervision, L.C. All authors have read and agreed to the published version of the manuscript.

Funding: The research was funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.3—Call for tender No. 341 of 15 March 2022 of Italian Ministry of University and Research funded by the European Union—NextGenerationEU. Project code PE00000003, Concession Decree No. 1550 of 11 October 2022, adopted by the Italian Ministry of University and Research, CUP E63C22002030007, Project title “ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON Foods.”.

Acknowledgments: All postbiotic products utilized in the study were a kind gift from Science Power SrL, Via Senato, 8, 20121 Milan (Italy). Science Power had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Conflicts of Interest: None reported.

Abbreviations

The following abbreviations are used in this manuscript:

iPB	Innovative postbiotic
ZO-1	<i>Zonula occludens-1</i>
MUC-2	Mucin-2
HBD-2	Beta-Defensin 2
LGG	<i>Lactocaseibacillus</i> GG
LGGp	<i>Lactocaseibacillus</i> GG postbiotic

Lpp	<i>Lactocaseibacillus paracasei</i> NPB-01 postbiotic
qRT-PCR	Quantitative real-time PCR
GUS-B	Glucuronidase beta
TGF-β	Transforming Growth Factor-β
RA	Retinoic Acid
TSLP	thymic stromal lymphopoietin
DCs	dendritic cells

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