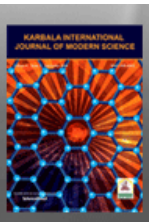

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Muramyl peptide blend ameliorates intestinal inflammation and barrier integrity in Caco-2 cells

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Abstract

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Keywords

muramyl peptides, adherence junctions, E-cadherin, TNF-α, autophagy, gut inflammation

RESEARCH PAPER

Muramyl Peptide Blend Ameliorates Intestinal Inflammation and Barrier Integrity in Caco-2 Cells

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Abstract

Intestinal barrier function depends on epithelial adhesion, which restricts permeability and microbial invasion from the internal environment. Impairment of barrier integrity and gut function is closely linked to pro-inflammatory changes. Inflammation is often the primary factor that provokes gut function disorders. The anti-inflammatory potential of postbiotics has been reported in recent years. Muramyl peptides (MPs) are small signaling molecules that stimulate intracellular pathogen receptors and can regulate cell responses. However, the molecular mechanisms of MPs' effects on intestinal cells remain unknown. The study of MPs treatment on lipopolysaccharide (LPS)-challenged Caco-2 intestinal cells aimed to investigate the postbiotic effects on intestinal barrier integrity, autophagy, and inflammation. An in vitro inflammation model was constructed using Caco-2 cells exposed to 10−100 μg/mL of LPS. The results showed a dose-dependent decrease in cell viability and E-cadherin content. Conversely, TNF-α content was increased, confirming proinflammatory disturbances. No significant differences were detected in cells exposed to 5−50 μg/mL doses of MPs. However, treatment with 50 μg/mL MPs ameliorated TNF-α production in LPS-exposed cells. The application of 20 and 50 μg/mL MPs exhibited a cytoprotective effect on cell viability in a dose-dependent manner. Furthermore, the 50 μg/mL dose of MPs ameliorated the reduction of E-cadherin content induced by LPS exposure. MPs treatment did not modulate the conversion of the autophagosome marker LC3-I into LC3-II but upregulated its content in LPS-challenged cells. These results demonstrated that MPs may be a promising tool to restore inflammatory balance and maintain intestinal barrier function.

Keywords: Muramyl peptides, Adherence junctions, E-cadherin, TNF-α, Autophagy, Gut inflammation

1. Introduction

The general mechanism that separates both human and animal bodies from the external environment is constructed with various epithelial cell types, including gastrointestinal epithelium. The monolayer of intestinal epithelial cells is not the only mechanism providing barrier function; gut immunity mechanisms also play an important role in maintaining this barrier. Metabolic diseases, gut infections, and various toxins can affect epithelial cells and cause an imbalance in permeability-barrier functions, known as the "leaky gut" phenomenon [1]. Sustained gut function requires the stimulation of barrier integrity, which is the most critical mechanism for maintaining intestinal health. Adhesive junctions between neighboring epithelial cells form a physical barrier that prevents the infiltration of pathogens and toxic compounds. Another important function of the intestinal epithelium is the translocation of nutrients through both the apical and basolateral membranes of epithelial cells [2].

The transport of nutrients and paracellular permeability are two intestinal functions regulated by epithelial cell adhesion. The efficiency of the intestinal barrier depends on the physical structures of the mucosa and epithelial cells, with intercellular adhesion playing an essential role [3]. Intercellular adhesion molecules form intercellular junctions, restrict the distance between epithelial cells, and maintain barrier integrity [4]. The two main groups of adhesion proteins that form the physical foundation of the

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barrier function are tight junction and adherence junctions [5–7]. Among tight junction proteins, occludin is one of the most studied and is considered a molecular biomarker of intestinal barrier alterations [8].

These features of tight junction were confirmed by results showing various barrier disturbances accompanied by downregulation of occludin expression [9,10]. E-cadherin, a member of the adherence junctions proteins, is described as an epithelial-specific cell adhesion molecule [11]. A decrease in both E-cadherin and occludin expression has been associated with epithelial barrier disruption [12]. Treatment with sphingosine has been shown to cause an increase in E-cadherin content in adherence junctions, thereby improving epithelial barrier integrity [13].

Infectious agents are the primary cause of gut barrier destruction. Recently, changes in E-cadherin content were observed in a model of coronavirus-induced intestinal barrier disturbance [14]. An increase in the soluble form of E-cadherin was reported in a study of Caco-2 cell cultures infected with the coronavirus SARS-CoV-2 [15]. *Eimeria* invasion has also been identified as a causative factor of intestinal barrier disruption, leading to the downregulation of E-cadherin and claudins [6]. Despite separate data linking adherence junctions protein expression to gut disturbances, the specific role of E-cadherin in maintaining intestinal barrier integrity remains unclear [16].

Various factors can initiate intestinal inflammation [17,18]. Inflammation is often associated with cytokine overproduction and release, which induces imbalances in multiple signaling pathways, including the activation of nuclear factor kappa B-cells (NF-kB) [19]. Additionally, pro-inflammatory changes can lead to disturbances in epithelial cell junctions and a reduction in intercellular adhesion [20]. Both chronic and acute inflammation have been confirmed as initiators of intestinal injury [21]. The integrity of the intestinal barrier and the regulation of inflammation are key components of gut health. Like other tissues, gut inflammation stimulates cytokine release, macrophage and lymphocyte infiltration, and an antimicrobial pro-inflammatory response [22,23]. While these inflammatory cascades offer protection, they can also lead to host cell disruption due to excessive cellular responses. Disruptions in intestinal barrier integrity have been reported in numerous in vivo and in vitro studies involving various causative factors [21,23,24]. Therefore, anti-inflammatory agents could be a promising strategy to protect gut health against intestinal barrier disorders.

There is increasing evidence that several microbial metabolites can modulate gut barrier function through key pathways associated with cellular response [25]. Probiotics, prebiotics, parabiotics, and postbiotics have been tested individually and/or in combination to treat gut abnormalities in recent decades [26–28]. Microbial metabolites retain their bioactivity despite exposure to elevated temperatures and/or enzymatic cleavage [29]. Thus, postbiotics, which are isolated in various ways from bacteria, can exhibit protective effects against pathogens and promote intestinal health [30,31]. Additionally, recent data suggest that postbiotics extracted from *Saccharomyces boulardii* exhibit protective efficacy against colitis in mice, similar to live bacteria [32]. Postbiotics from Lactobacillus have demonstrated more extensive immunomodulatory activities than their live strains [33].

Peptidoglycan fragments, known as MPs, are microbial wall-derived postbiotics that exhibit significant potential to regulate host cell responses against pathogen invasion [34]. Moreover, MPs are signaling molecules recognized by the intracellular receptor nucleotide-binding oligomerization domain 2 (NOD2), which belongs to the pattern recognition receptors (PRR) family [35,36]. Upon stimulation by MPs, the NOD2 receptor activates cytokine expression and innate immunity [37,38,18]. The stimulatory effect of MPs has been demonstrated in various cell types [39,40]. However, there is no data available on the stimulation of intestinal epithelium by MPs. The potential to apply MP-based postbiotics in gut diseases has garnered significant interest in recent years [41]. Despite several reports on the beneficial effects of postbiotics, the exact mechanisms involved in promoting intestinal health remain unclear [42,43]. Lipopolysaccharide (LPS) is a well-known pro-inflammatory agent [44]. LPS triggers multiple effects, activating innate immune signaling and inducing intestinal barrier dysfunction [45,46]. Therefore, LPS-exposed epithelial cell cultures are a suitable model for assessing the efficacy of cytoprotective agents against inflammation-induced cytotoxicity [47–49]. The aim of the present study was to investigate the cytoprotective effect of MPs on inflammation and intestinal barrier function in LPS-challenged intestinal cells.

2. Ethical approval

The study using human cell culture was carried out within the framework of the “General Ethical Principles of Animal Experiments”, which have been approved by the National Congress of Bioethics held in Kyiv in 2001, and in a line with the requirements of

the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (ETS No.-123; https://www.coe.int/en/web/cdcj/laboratory-animals). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Dnipro State Agrarian and Economic University (DSAEU), Ukraine (Date, December 5, 2023, No. 11723).

3. Materials and methods

3.1. Isolation of muropeptide fraction and its amino acid composition analysis

Proteoglycan fragments were obtained through lysozyme lysis of the *Lactobacillus delbrueckii* cell wall according to the protocol of ENZIM Group Biotechnology Company ("Enzim" Ltd, Ukraine), as described earlier [40]. The amino acid composition of the muropeptide fraction was analyzed using acid hydrolysis. In summary, the muropeptide fraction sample with a concentration of 1 mg/mL was mixed with an equal volume of 6 M hydrochloric acid (HCl) in a glass container. The hydrolysis was performed in a nitrogen atmosphere at 110 °C for 24 h. The hydrolyzed product was neutralized with 6 M sodium hydroxide (NaOH) and then diluted with 0.02 M HCl. The amino acid composition was determined using an amino acid analyzer, Hitachi L-8800 (Hitachi Corp., Japan).

3.2. Cell culture and treatment

The human Caucasian colon adenocarcinoma (Caco-2) cell line was purchased from the American Type Culture Collection (HTB-37™, ATCC, Manassas, VA). The growth of Caco-2 cells was carried out in 75 cm^2 flasks in Dulbecco's Modified Eagle Medium and Hank's F12 medium (1:1) (DMEM/F12 contained high glucose, 4500 mg/dL; HyClone, Invitrogen Company, USA), supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 1% sodium pyruvate, 1% glutamine, and 1% streptomycin/penicillin. The cell culture was incubated in a humidified atmosphere at 37 °C and 5% CO_2, with the medium replaced every 48 h. When the cells achieved nearly 90% confluence, they were passaged using 0.25% trypsin–EDTA (0.25% trypsin, 0.02% EDTA). During the last passage, the cells were transferred to 6-well plates at a concentration of 5×10^5 cells/well and cultured until they reached 100% confluence. The cells at complete confluence were washed with phosphate-buffered saline (PBS) and cultured in medium containing 0.2% FBS for 12 h before treatment. Caco-2 cells were exposed to LPS (10, 50, and 100 μg/mL), MPs (5, 20, and 50 μg/mL), and a combination of LPS (50 μg/mL) with various doses of MPs. The control group was treated with an equal volume of dimethyl sulfoxide (DMSO).

LPS exposure was conducted to simulate intestinal inflammation (Cat. n. L2630, Merck, Germany). Confluent Caco-2 cells were exposed to LPS at doses of 10, 50, and 100 μg/mL by adding a stock solution of LPS, dissolved in DMSO at a concentration of 1 mg/mL, immediately before exposure. Confluent Caco-2 cells were also exposed to MPs at final concentrations of 5, 20, and 50 μg/mL by adding an MP stock solution prepared by dissolving it in DMSO at 1 mg/mL immediately before exposure. The cells were pre-treated with MPs for 120 min, after which an LPS dose of 50 μg/mL was added to the medium for the MPs + LPS co-exposed cells.

3.3. Cell viability (MTT assay)

The effect of MPs on the viability of Caco-2 cells was assessed using an MTT assay kit (Bahuguna et al., 2017). Briefly, Caco-2 cells (1×10^4 cells/well) were seeded in 96-well plates with DMEM/F12 and incubated for 24 h at 37 °C, 95% humidity, and 5% CO_2. After attachment and the onset of growth, the cells were washed with PBS and exposed to the aforementioned doses of MPs, LPS, and LPS + MPs for 48 h. Subsequently, the medium was replaced with fresh medium containing 10 μL of MTT reagent and incubated for 4 h. Then, dimethyl sulfoxide (DMSO, 100 μL) was added into the wells and incubated for 10 min. The optical density values were measured at 570 nm in the presence of Sorensen's buffer using an ELISA plate reader (SpectraMax 384 Plus, Molecular Devices, USA).

3.4. Western blot analysis

Caco-2 cells were cultured to achieve a confluent monolayer and exposed as previously described. After 48 h of exposure, the control group and the groups treated with LPS, MPs, and LPS + MPs were washed with cold PBS and harvested by scraping, without trypsinization. The cells were centrifuged and lysed with RIPA buffer containing a protease and phosphatase inhibitor cocktail (Cat. n. 20–180, Cat. n. PPC1010, Merck, Germany). After lysis, the cell extracts were centrifuged at 40,000 g for 20 min. The total protein content was measured using the modified Bradford method and Lowry assay [50]. The protein extracts were mixed with Laemmli sample buffer containing 0.1 M dithiothreitol in a 1:1 ratio and boiled for 5 min. The protein samples were

then frozen and stored at −80 °C before Western blot analysis was performed.

The proteins were separated with polyacrylamide gel (PAAG) electrophoresis using 5–20% gradient of acrylamide and transferred from the gel onto polyvinylidene fluoride (PVDF) membrane. After transferring, PVDF membrane was washed with Tris buffer saline contained 0.1% Tween-20 (TBS-T) and blocked in 1% BSA solution in TBS-T. Blocked membrane was probed overnight at 4 °C for primary antibodies anti-E-cadherin (1:1000, Santa Cruz, sc-271842), anti-TNF-α (1:1000, Santa Cruz, sc-52746), anti-MAP-LC3b (1:2500, Abcam, ab48394), and anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibodies as a housekeeping marker (1:2000, Santa Cruz, sc-365062). After washing, the membrane was incubated with secondary anti-mouse IgG antibodies conjugated with horseradish peroxidase at room temperature during 60 min (1:10000, Abcam, ab6721). Immunostaining was performed with use luminol-coumaric acid-hydrogen peroxide solution by the enhanced chemiluminescence method. Densitometric analysis carried out using TotalLab TL120 software (USA).

3.5. Statistics analysis

All experiments were performed in triplicate. The arithmetic mean (M) and standard error (SE) were used to present the quantitative data. Statistical comparisons were performed using one-way analysis of variance (ANOVA) with StatView 5.0 (SAS Institute Inc., USA). The graphs were created using GraphPad Prism 9 software (GraphPad Software, USA). P-values less than 0.05 were considered statistically significant.

4. Results and discussion

4.1. Amino acids content of MPs blend

The amino acid content in the obtained MPs fraction was measured to verify the types of adherence junctions or MPs fragments produced using lysozyme. The data demonstrated that the amino acid composition and the ratio of prevailing components were similar to the previously reported muramyl pentapeptide structure [34,40]. The content of alanine was higher than that of the other detected amino acids (Table 1).

4.2. Cell viability

The analysis of cell viability was carried out to reaffirm the LPS-induced disturbance and assess the effects of MPs on the intestinal cells. The observed results showed a dose-dependent decline in cell viability caused by LPS exposure compared to untreated cells (Fig. 1).

No statistical changes were detected in cell viability in the MPs-exposed group in compare with the control unexposed cells. In contrast, an increase in cell viability was observed in the LPS + MPs-exposed cells compared to the LPS-exposed group.

4.3. TNF-α content

The content of TNF-α was measured to reaffirm pro-inflammatory changes in the LPS-induced cell model. A statistically significant increase in TNF-α content was observed in Caco-2 cells treated with LPS compared to the control (Fig. 2). The decrease in TNF-α production was observed in Caco-2 cells exposed to LPS + MPs compared to LPS-exposed cells. Conversely, a slight increase in TNF-α content was detected in MPs-treated cells compared to the control group (Fig. 2).

4.4. E-cadherin content

E-cadherin content was measured to estimate the effect of MPs on the intercellular adhesion of the intestinal epithelium. Observed results demonstrated a statistically significant decrease in E-cadherin content in LPS-exposed cells (Fig. 3).

4.5. Autophagy flux

The level of E-cadherin in the MPs-exposed group was similar to that of the control group. However, the content of E-cadherin in LPS + MPs-exposed cells was improved in compare with LPS-treated cells. The autophagosome marker LC3 was detected to examine the impact of MPs on the modulation of the cytoprotective effect in LPS-challenged cells. LPS exposure decreased the relative content of LC3-II, along with a moderate increase in the LC3-II/LC3-I ratio (Fig. 4).

4.6. Multiple MPs effects

MPs exposure statistically increased LC3-II/LC3-I ratio in 5 and 20 μg/mL doses while 20 and 50 μg/mL

Table 1. Amino acid content in peptidoglycan fragments isolated from L. delbrueckii.

Amino acid	Ala	Asp	Lys	Glu	Gly
Content, μg/mg	15.03	4.12	10.24	8.79	5.25

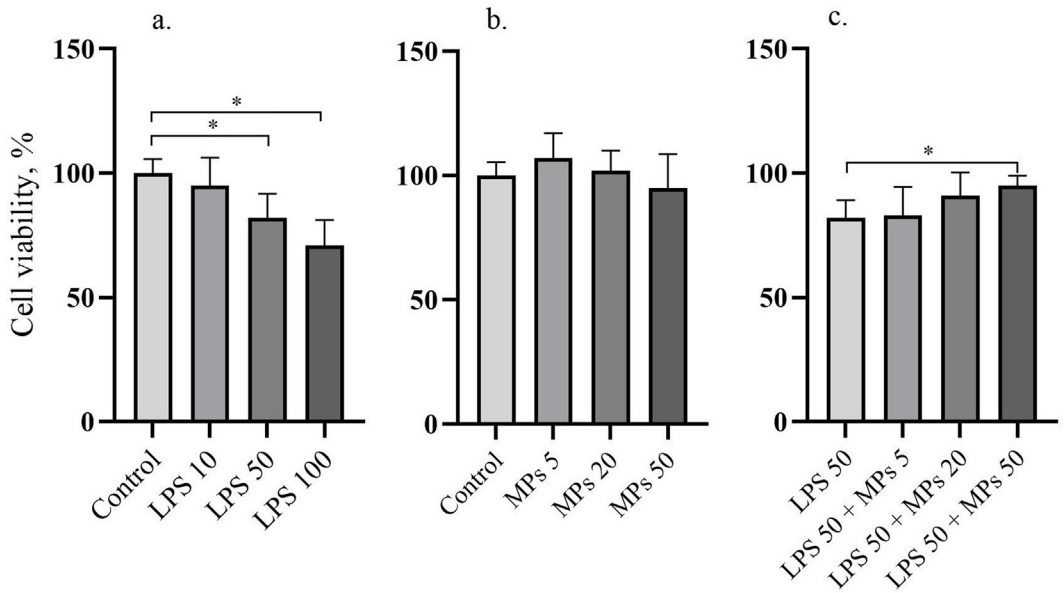

*Fig. 1. Cell viability level in Caco2 cell exposed to LPS (a), MPs (b) and LPS + MPs (c). Data are shown as Mean ± SEM of four independent experiments for every exposure (n = 4). Significant of differences represented as: * - P < 0.05.*

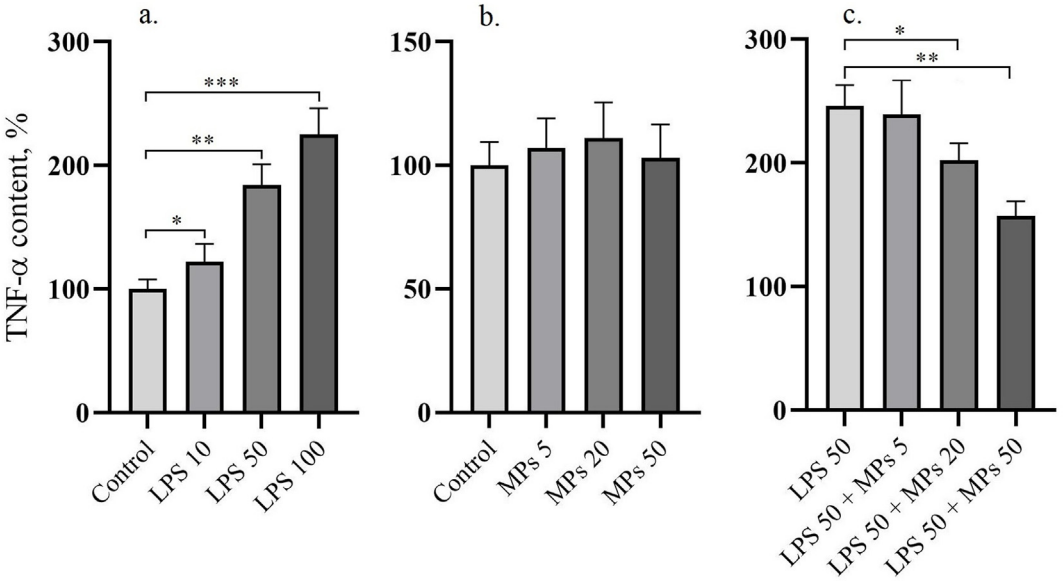

*Fig. 2. The content of TNF-α in Caco2 cell exposed to LPS (a), MPs (b) and LPS + MPs (c). Data are shown as Mean ± SEM of four independent experiments for every exposure (n = 4). Significant of differences represented as: * - P < 0.05, ** - P < 0.01, *** - P < 0.001).*

doses initiated dramatic increase in both total LC3 and LC3-II/LC3-I ratio (Figs. 4 and 5).

Thus, MPs treatment induced significant autophagy flux in LPS-challenged cells. Jointly, the obtained results provide evidence that MPs application exhibits a protective effect against LPS-induced inflammation and its complications, while the anti-inflammatory effect is accompanied by the amelioration of proteins and the initiation of autophagy flux.

4.7. Modern strategies to maintain intestinal health

Strategies for promoting intestinal health remain a significant concern for both humans and animals. A large number of pathogens and detrimental factors can disrupt gut function and consequently induce injury in other tissues [3]. Various adverse stimuli, including inflammation, can provoke the destruction of epithelial cell junctions, initiating barrier dysfunction [51]. The multiple harmful effects of

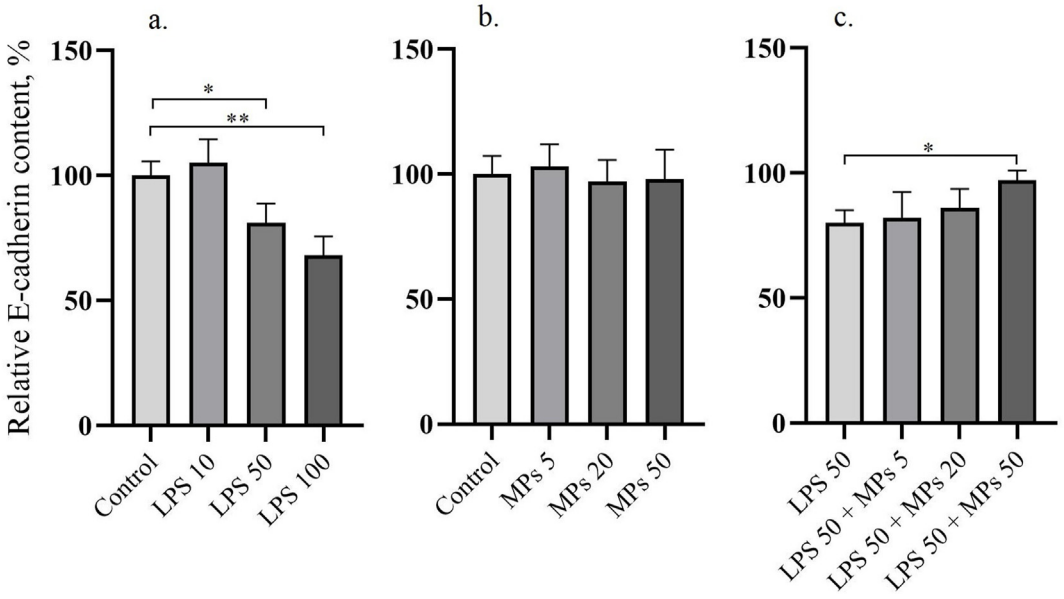

*Fig. 3. The content of E-cadherin in Caco2 cell exposed to LPS (a), MPs (b) and LPS + MPs (c). Data are shown as Mean ± SEM of four independent experiments for every exposure (n = 4). Significant of differences represented as: * - P < 0.05, ** - P < 0.01.*

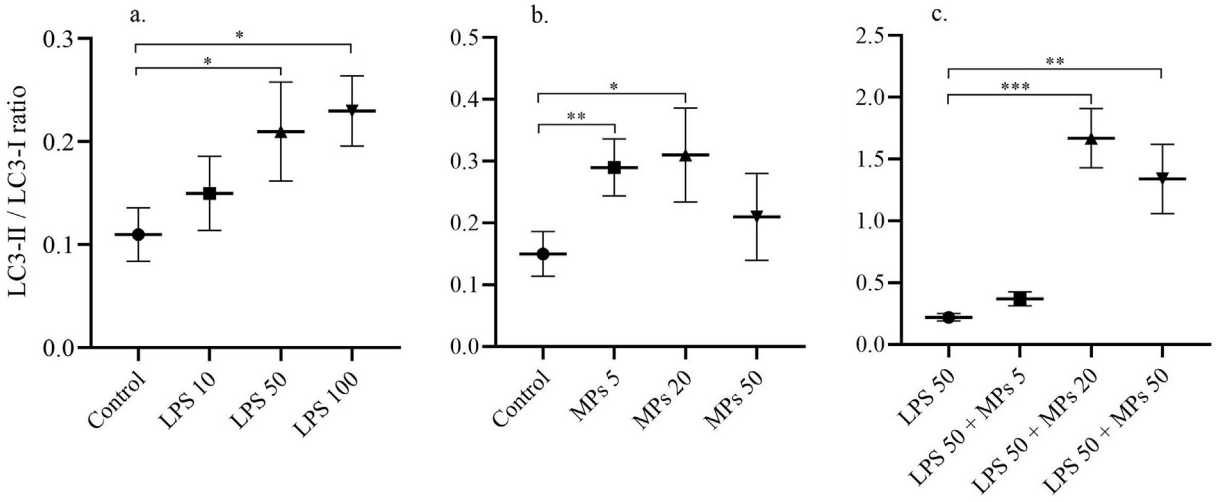

*Fig. 4. The LC3-II/LC3-1 ratio in Caco2 cell exposed to LPS (a), MPs (b) and LPS + MPs (c). Data are shown as Mean ± SEM of four independent experiments for every exposure (n = 4). Significant of differences represented as: * - P < 0.05, ** - P < 0.01, *** - P < 0.001).*

inflammation on the intestinal barrier are confirmed by numerous reports [52,53,49]. Despite recent advancements in the study of dietary factor supplementation to maintain intestinal function, the exact mechanisms behind gut disruption, commonly referred to as "leaky gut," are still poorly understood [52].

In recent decades, postbiotic supplementation has been described in several reports and is considered a promising tool to alleviate metabolic diseases [42,43]. Recent data indicate that microbiome-derived molecules, including peptidoglycan fragments, can improve neuroepithelial cell functions [20]. However, the protective effect of postbiotics on the intestinal epithelium remains elusive. Furthermore, the molecular mechanisms behind the application of commercial MP-based postbiotic drugs are also unknown [54].

The present study aimed to clarify the protective effect of MPs on gut barrier integrity and the suppression of intestinal inflammation. Considering that LPS exposure simulates intestinal mucositis, the observed decline in cell viability and TNF-α upregulation in our study reaffirm the validity of the

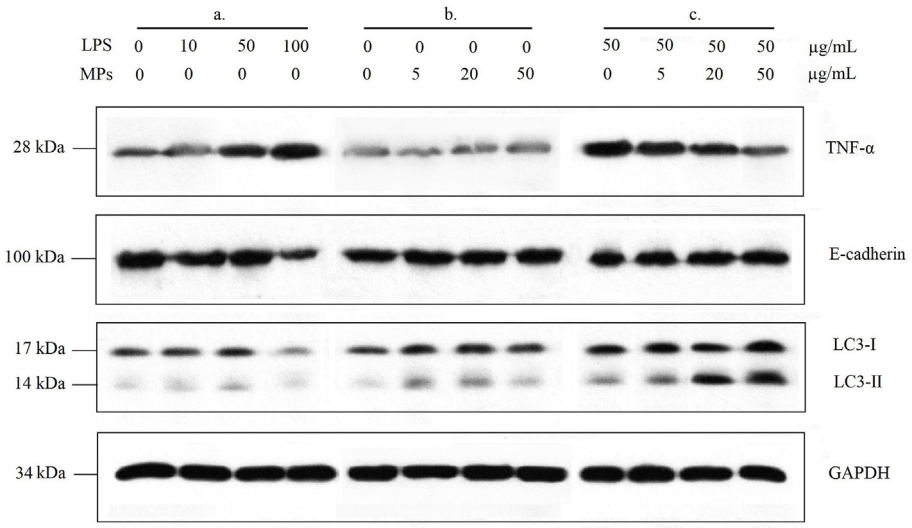

Fig. 5. Western blot results of TNF-α, E-cadherin, LC3, and GAPDH analysis. Caco2 cells were exposed to LPS - 10–100 μg/mL, MPs - 5–50 μg/mL, and combined exposure LPS (50 μg/mL) with MPs (5–50 μg/mL).

applied in vitro model of intestinal cell injury [2,46,49]. Additionally, the present data show that the LPS-induced decline in cell viability was paralleled by TNF-α upregulation, reflecting the association between inflammation and intestinal disturbance.

Inflammation is confirmed to be the leading initiator of intestinal epithelial lesions. The primary role of the inflammatory response is the local activation of innate immunity against infectious agents [51]. However, cytokine overproduction can create an imbalance in regulatory pathways and induce injury to host cells through an extensive pro-inflammatory response [47]. On the other hand, anti-inflammatory agents can ameliorate inflammatory damage by suppressing the production of pro-inflammatory cytokines, thereby alleviating gut inflammation [51,49]. The present results on LPS-induced increases in the pro-inflammatory cytokine TNF-α demonstrated a dose-dependent inflammation in Caco-2 cells. Therefore, the upregulation of TNF-α production may be linked to a decline in cell viability as well as intestinal function.

4.8. The role of epithelial intercellular adhesion proteins in intestinal barrier integrity

Intestinal barrier disruption is closely related to epithelial cell lesions [52]. Conversely, epithelial barrier integrity depends on the efficiency of epithelial intercellular adhesion [32]. Notably, desmosomes, tight junctions, and adherens junction proteins together constitute cell–cell contacts that maintain the intestinal barrier [55]. E-cadherin-mediated cell–cell interactions form a special type of adhesion complex that is linked to cytoskeletal structures. Dimers of E-cadherin bind to the extracellular domains of each other on neighboring cells, while the intracellular domain of E-cadherin binds to catenins [11]. The catenins form a connection to the actin cytoskeleton, which provides signal transduction from intercellular junctions to intracellular regulatory pathways [16,56]. Thus, abnormalities in E-cadherin content can initiate an imbalance in vital epithelial cell pathways, accompanied by both pro-inflammatory changes and barrier disruption. The observed results regarding the LPS-induced decline in E-cadherin content align with published findings on epithelial barrier disruption [57]. Therefore, E-cadherin downregulation may be mediated by pro-inflammatory changes, which consequently initiate barrier breakdown, at least partially due to adherence junctions deficiency.

Intestinal cell responses to MP exposure may be associated with the initiation of autophagy. Recent data suggest that muramyl dipeptide (MDP) exposure attenuates intestinal inflammation, modulates autophagy flux, and improves intestinal barrier integrity and autophagy stimulation in the intestinal epithelium [35]. NOD2 is considered an initiator of autophagy flux through the activation of autophagy-related protein 16-like protein 1 (ATG16L1) in response to both bacterial invasion and inflammatory disorders [58,59].

4.9. Autophagy flux supports intestinal functions

Autophagy is a mechanism that clears the cytoplasm of damaged proteins and intracellular pathogens by packaging them into vesicles, fusing these vesicles with lysosomes, and degrading the

phagosome-engulfed cargo to generate reusable molecules [60]. Autophagy plays a crucial role in the cellular stress response and helps maintain cell survival [61]. An upregulation of autophagy flux increases resistance against detrimental factors. The stimulation of autophagy is described as an indirect factor in tight junction regulation and, consequently, in the functioning of epithelial cells [61,62]. Despite the absence of direct evidence linking postbiotic effects to autophagy upregulation, the obtained results demonstrate that the protective effect of MPs may be mediated by the stimulation of autophagy via NOD2-dependent pathways. Furthermore, our results show that MPs alleviate LPS-induced TNF-α increases in parallel with a decline in E-cadherin content and modulation of the autophagosome marker LC3. These data align with results reported by other authors regarding the modulation of TNF-α, E-cadherin, and autophagy flux in intestinal epithelial cells exposed to postbiotics [35,6,63,64].

4.10. Mechanisms of the postbiotic protective effect

Despite the aforementioned results, contradictory data regarding MDP exposure have been demonstrated. LPS-challenged rats treated with 1.5 and/or 15 mg/kg doses of MDP showed that only the 15 mg/kg dose induced a significant increase in the pro-inflammatory cytokine IL-6 content. Additionally, MDP exposure was found to exacerbate inflammatory damage in both intestinal and lung tissues and initiate autophagy suppression [65]. Thus, these data suggest that oral administration of MDP can induce a different epithelial cell response compared to in vitro cell treatment. On the other hand, studies on the transport of muropeptide precursors indicate that MPs can serve as indirect regulators of intestinal barrier strengthening and help maintain host immune homeostasis [41]. Moreover, recent results from in vivo and in vitro studies have demonstrated that MDP supports epithelial barrier repair by upregulating both tight junction and adherens junction protein expression, reducing apoptosis, and initiating autophagy flux [58].

Taking into the account that MDP is the smallest peptidoglycan fragment confirmed as an activator of the NOD2 receptor, the present results on the effects of the MPs blend could depend on the activation of alternative pathways related to the MDP mechanism. Furthermore, our results pertain to Caco-2 cells challenged with a blend of LPS and MPs, in which muramyl pentapeptide is the prevailing component and has a different structure compared to MDP. Postbiotics containing peptidoglycan from *Lactobacillus rhamnosus* have demonstrated immunoregulatory effects similar to those of live *L. rhamnosus* probiotics [66]. This data indicates that peptidoglycan is a key component of both postbiotics and probiotics derived from *Lactobacillus*. Additionally, another postbiotic composition of *L. rhamnosus* has been reported as a tool to protect against LPS-induced dysfunction in intestinal barrier integrity through the upregulation of zonula occludens-1 [67].

The adherence junctions or mechanism for providing innate immunity stimulation against various bacterial targets involves pattern recognition receptors (PRRs). The most common fragments of the bacterial wall are muropeptide fragments (MPs), which are specific ligands for the NOD2 receptor. Recent studies have confirmed both the modulation of innate immune activity and the upstream signaling of NOD2 by microbial invasion into the cytosol [68]. NOD2 is one of the intracellular PRRs that recognizes peptidoglycan fragments as signals of potential bacterial invasion. The anti-inflammatory effect of MPs may be related to the cascade activation of innate immunity, with NF-kB playing a key role in cytokine upregulation [19,37].

Peptidoglycan fragments are involved in the signaling of microbiome-host communication through their production in the microbiome and translocation into host cells [34]. The signaling properties of MPs depend on the biology of the bacterial wall. The turnover of peptidoglycan fragments is a continuous process in the bacterial wall, accompanied by the excision of muropeptides as soluble fragments [43,69]. The released soluble fragments can be reabsorbed by bacterial cells and reused for bacterial wall restoration [34,70]. Another portion of MPs is absorbed by epithelial cells, where this postbiotic serves as a signaling factor for host cells. The most effective way to modulate intestinal cell responses is by stimulating the PRRs responsible for controlling microbial invasion [71]. Peptidoglycans are also confirmed as signaling molecules that induce cellular stress responses against microbial invasion [72,34]. The response stimulated by peptidoglycans leads to an increase in metabolic activity and the viability of host cells. The production of peptidoglycans in the gut by microbial muramidase enhances the antioxidant system, cytokine expression, and body weight gain in broiler chickens [73].

Additionally, the effect of MPs may be mediated by hexokinase II [34]. Hexokinase II acts as an intracellular sensor for MPs, modulating mitochondrial activity [36]. Considering that several regulators of autophagy are mitochondrial-associated proteins,

the results regarding MPs-dependent autophagy stimulation suggest that hexokinase II could be involved in the cytoprotective effect of this postbiotic. The data show that the LPS-induced decline in cell viability was alleviated by MPs treatment. Thus, the observed results indicate that the application of MPs develops a cytoprotective effect, particularly concerning the viability of epithelial cells. Furthermore, the improvement in cell viability was accompanied by anti-inflammatory and barrier-maintaining effects.

The MPs-dependent activation of autophagy may play a crucial role in all these effects by stimulating the synthesis of new E-cadherin molecules while cleaving damaged ones. The present results demonstrate differing effects of MPs on LPS-unexposed versus LPS-exposed Caco-2 cells. Non-significant differences in cell viability, TNF-α, and E-cadherin content were observed in cells exposed to 5, 20, and 50 μg/mL doses of MPs. However, all these parameters were alleviated in LPS-exposed cells that were additionally treated with a dose of 50 μg/mL MPs (Figs. 1–4). The results we obtained regarding the protective effect of MPs on gut barrier integrity are presented for the first time. Thus, the application of MPs can develop a protective effect through the decrease of TNF-α content, upregulation of E-cadherin, and stimulation of autophagy flux. Furthermore, the present data are in concordance with previously reported findings on the link between pro-inflammatory changes and disturbances in gut barrier integrity [74,58,41]. Additionally, our results support the hypothesis regarding the multiple effects of postbiotics in modulating the balance of cytokine production, intercellular adhesion, and programmed cell death [75,58].

The application of MPs-based postbiotics for treating enteric diseases has gained special attention in the last decade. The complex composition of postbiotics results in multiple effects on maintaining intestinal health [76]. Moreover, the various methods used to produce feed supplements allow for the creation of diverse products with specific bioactivities [77]. Recent data have shown that postbiotics exhibit protective effects comparable to those of probiotics, with extended shelf life and safety advantages. The anti-inflammatory effect of postbiotics is hypothesized to be one of the most important factors contributing to their cytoprotective properties [58]. The obtained results can serve as a foundation to support or refute this hypothesis. Given the observed potential of MPs to ameliorate inflammation, enhance cell viability, maintain epithelial barrier integrity, and stimulate autophagy flux, the present results align with this hypothesis.

5. Conclusion

The present data demonstrate that MPs blend inhibit proinflammatory TNF production in LPS-treated intestinal cells. MPs application ameliorate cell viability and adherence junctions protein content in the Caco-2 cells affected with LPS. The observed cytoprotective effect of MPs can be related to differential stimulation of intracellular PRRs that recognize the peptidoglycan fragments and mediate the regulation of innate immunity. The potential of MPs to restore intestinal barrier after inflammatory-caused injury may offer a promising approach to protect gut health against other detrimental factors as well. The further study is required to explore cytoprotective effect of this postbiotic on intestinal health.

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