

The effect of hydrolysates of proteins from rice milk on the physiological response of enterocytes and on the adhesion of bacteria from healthy and allergic people – an *in vitro* study

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Abstract

Designing an optimal diet requires knowledge of the biological activity of food products, particularly in relation to people with food allergies. The hypothesis, which constitutes the basis of this thesis, states that the peptides and glycopeptides released from proteins by enzymatic hydrolysis are able to change the quantity and quality of the human gastrointestinal ecosystem. Such substrates may interfere with adhesion to the intestinal epithelium microbiota and alter enterocytic metabolic activity. The aim of this study was to determine the effect of protein hydrolysates from rice milk substitute on gut epithelial cells and the intestinal microbiota of healthy people and ones suffering from an allergy to milk. The following experimental work applied systems that reflect the conditions occurring in the gastrointestinal tract.

Key words: rice milk, milk substitute, allergy to cow's milk, intestinal bacteria, bacterial adhesion, intestinal ecosystem.

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Introduction

A correctly balanced diet makes a major contribution to the wellbeing and healthy ageing of humans. This is especially relevant in the case of people suffering from complex diseases, such as food allergy, involving diverse elements that constitute the intestinal ecosystem. Food allergy is a complex disease, influenced by phylogenic genes and environmental factors, and it represents an increasing concern to society with no cure available to date for this illness [1]. It is defined as an inappropriate immunological response to normally harmless food components. Allergy to milk proteins is one of the major allergies, and even though it tends to diminish with age, it still concerns 1-2% of adults [2]. Since milk and milk proteins are consumed not only on their own but also serve as ingredients in a wide spectrum of food products, such as bakery and meat products, sweets, etc., diets eliminating milk may negatively impact the quality of life and economy of food allergic consumers' daily life. Adult people tend to look for beverages that may substitute cow's milk in their diet, such

as rice milk. This product contains no lactose and milk proteins, which makes it potentially attractive for people with intestinal disorders and an allergy to milk proteins. Despite the consumption of milk substitutes such as rice milk, knowledge on the impact of its protein fractions on the intestinal ecosystem is still very limited.

Designing an optimal diet requires knowledge of the biological activity of food products, particularly in relation to people with food allergies. A properly functioning intestinal ecosystem, which consists of microbiota and epithelium along with the immune system, plays an important role in maintaining the physiological equilibrium of the human body [3]. The small intestine is a critical niche of interplay between the external factors, such as food, and the internal environment constituting the human organism. Phenomena related with the interactions between the type of consumed diet, host cells, and autochthonic microbiota of the gastrointestinal tract (GIT) have been studied so far only partially. However, consumed food substrates undergo modifications due to enzymatic hydrolysis by proteinases present in the

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GIT. The enzymatic degradation may lead to the alteration of biological activities displayed by initial protein fractions due to the release of highly bioactive peptides and glycopeptides [4]. Such peptides may exert antibacterial, immunomodulatory, opioid-like, and hormone-like properties, etc., thus their effects are multiple but not classically nutritional [5]. Therefore, alterations in terms of the biological activity of food components caused by the hydrolysis process have to be taken into account while constructing the diet recommendations [6]. Peptides and glycopeptides released by the hydrolysis of proteins may significantly modulate the condition and activity of the intestinal ecosystem. Such substrates may interfere with adhesion of microbiota to the intestinal epithelium, and alter enterocytic metabolic and proliferative activity and the level of secretion of proinflammatory cytokines [7], thus influencing the intestinal barrier functionality and integrity and consequently determining the health-status of the consumer. This is especially relevant in terms of people with an intestinal imbalance that occurs when a food allergy is present. Therefore, the aim of this study was to determine the effect of protein hydrolysates from rice milk substitute on the physiological response of the model of the small intestinal barrier of healthy and allergic people. The small intestinal barrier was imitated by co-culture of Caco-2 cells, representing enterocytes, HT-29-MTX cells, standing for Goblet cells and bacteria immobilised to their surface. To address the scientific problem, bacterial representatives of the small intestinal region, such as lactobacilli, enterococci, and enterobacteria, were obtained from the faeces of healthy volunteers and ones suffering from an allergy to cow's milk, and they were used to build up models of the small intestinal barrier. Subsequently, the effect of hydrolysates from rice milk proteins exerted on the model was studied.

Material and methods

Material

A commercially available rice milk powder product containing (per 100 g of product) 2.6 g of proteins, 70 g of

carbohydrates, 21.3 g of fat, < 0.1 mg of cholesterol, and < 1 mg of gliadin was used/investigated in the study. It contained no lactose.

Protein extraction from raw rice milk substitute

Protein extraction was conducted according to the method described in the paper by Świątecka *et al.* [8].

Gastric and duodenal digestion of protein extract from rice milk

Extracted proteins were subjected to gastric and duodenal conditions according to the protocol previously described by Mandalari *et al.* [9].

Samples of hydrolysates used for experimental analysis were described as: RH – substrates upon pepsin-pancreatin hydrolysis of proteins from rice milk.

Determination of the degree of hydrolysis (DH) and determination of available amino groups and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The analysis was conducted as previously described in the paper by Świątecka *et al.* [8].

The intestinal barrier model

The small intestinal barrier model used in this study consisted of Caco-2 cells, representing enterocytes, HT-29-MTX cells, representing Goblet cells and intestinal bacteria, obtained from healthy and allergic people, which were adhered to the epithelial surface (Fig. 1). Such models were used to analyse the physiological response of epithelial cells (metabolic and proliferative activity, IL-8 secretion, transepithelial resistance, and gene expression) and the adhesive potential of bacteria to RH hydrolysates.

The experimental design

The study was divided into two modules: the adhesion experiment and the physiological response experiment (Fig. 2).

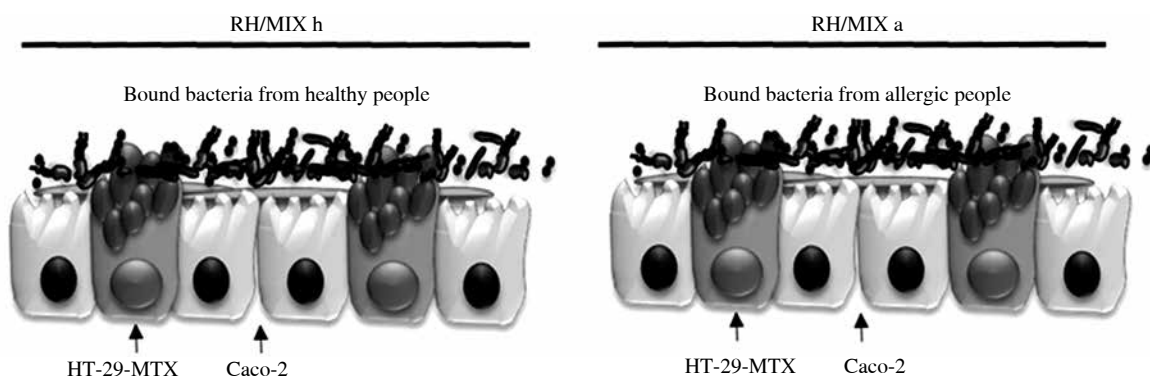


Fig. 1. Small intestinal barrier models used in the study

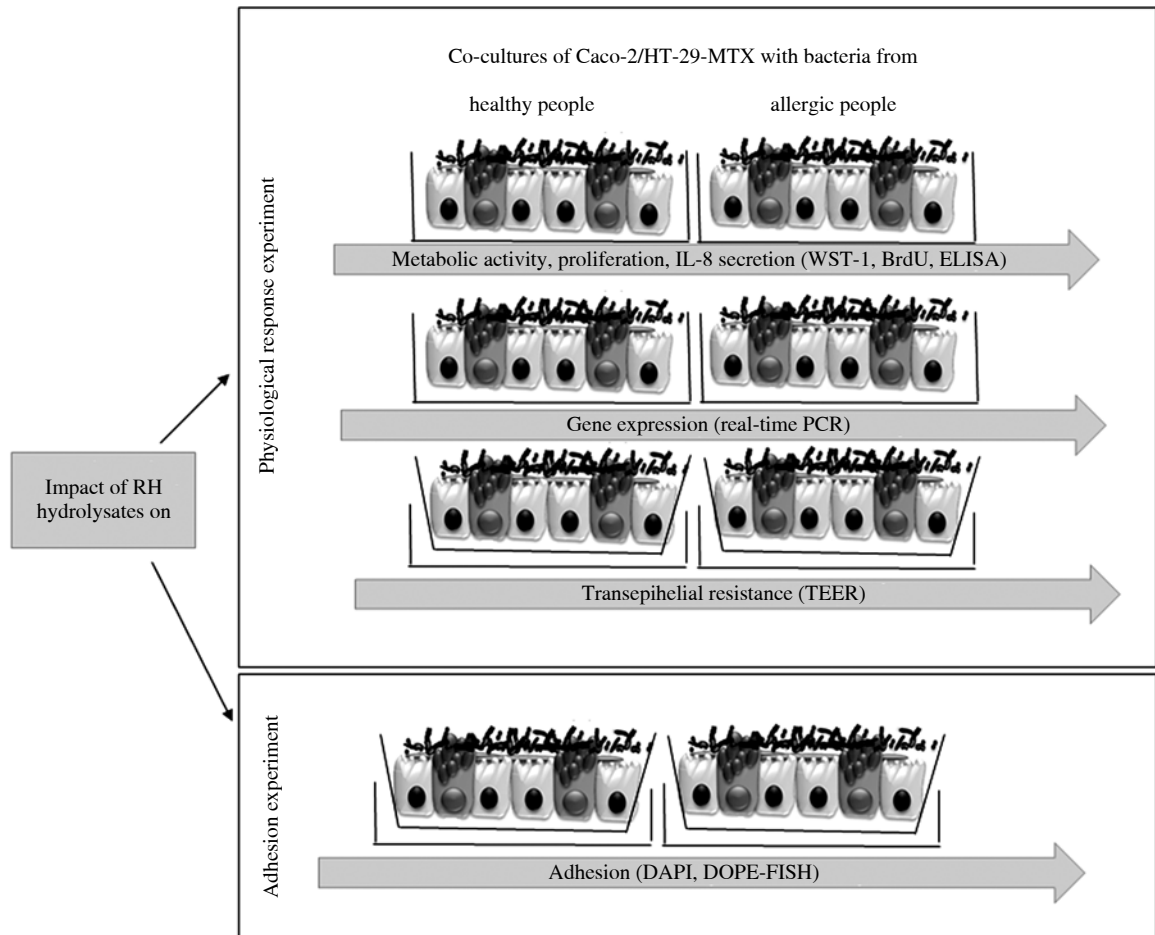


Fig. 2. The schematic experimental design of the study

Small intestinal barrier models used for the adhesion experiment were cultured in the insert cultures.

However, three separate co-cultures were used for the physiological response experiment: to study the metabolic and proliferative activity and IL-8 secretion (standard plate cultures); to study the gene expression (standard plate cultures); and to study the transepithelial resistance (insert plate cultures).

Bacterial isolates and growth conditions

Faecal samples were obtained from seven healthy people and seven persons suffering from gastrointestinal disorders after consumption of cow's milk. These volunteers were of both sexes at the age range of 18-55 years. Biological material was immediately frozen at -20°C until further analysis.

Faecal samples were transferred into an anaerobic workstation (Don Whitley Scientific), where they were suspended in sterile peptone water (ratio 1 : 10 w/v).

Subsequently, the samples were homogenised for three minutes with glass beads (5 mm diameter) and afterwards serially diluted 10-fold. 100 μl of particular dilution was transferred onto the solid media in order to obtain single colonies. Various solid media were used in the experiment in order to obtain particular bacterial isolate: Rogosa medium – for isolation of *Lactobacillus* (LAC), MacConkey medium – for Enterobacteriaceae (ENT), and KEA (kanamycin esculin agar) medium – for *Enterococcus* (ENTcc). These bacterial groups were selected as the main representatives of microorganisms colonising the human small intestine. Plates were incubated at a temperature of 37°C aerobically or anaerobically depending on the bacterial oxidative requirements. After incubation, single colonies, chosen on the macro- and microscopic basis, were collected and suspended in a sterile, anaerobic PBS buffer. From each medium (from seven volunteers) approximately 5-15 colonies were obtained and combined into one liquid culture. The cultures were centrifuged at 10,000 rpm for

five minutes, and the obtained pellets were resuspended in 1 ml of sterile, anaerobic PBS buffer. Such bacterial suspensions were used to prepare bacterial solutions (BS) as follows:

- heterogeneous-bacterial isolate solution (MIX): 70 µl of each bacterial isolate (ENT, ENTcc, LAC) was transferred into the 20 ml DMEM medium without antibiotics and used subsequently in the experiment.

Caco-2/HT-29-MTX cell co-culture conditions

Caco-2 is derived from the absorptive cells and therefore functions as a model of the small intestinal enterocytes [10], whereas HT-29-MTX stems from the goblet cells and thus mimics the mucus-producing intestinal cells. These two cell lines were used as a small intestinal model by being grown together in a co-culture, as described previously by Laparra and Sanz [5]. Both of them were obtained via Sigma Aldrich from the European Collection of Cell Cultures and were routinely cultivated in the filtrated DMEM medium (Dulbecco's Modified Eagle's Medium, Sigma) containing 20% of inactivated foetal bovine serum (FBS, Gibco), 1% of non-essential amino acids mixture (NEAA, Gibco), and 0.1% of penicillin-streptomycin solution (Sigma). The incubation was carried out at 37°C in 5% CO₂ atmosphere with humidity approximately 95%. Caco-2 cell line was used in passage number 47, whereas HT-29-MTX was used in passage number 53.

Caco-2 and HT-29-MTX were seeded together at a ratio of 9 : 1, to mimic the conditions in the small intestine [5]. For adhesion experiments and permeability study, the cells were seeded in dimensional cultures onto hanging inserts of pore size 0.4 µm and inner diameter 12 mm (Millipore, Merck, Poland), and placed in the 12-well tissue culture plates (Becton Dickinson) at a density 50,000 cells per cm². For the physiological response experiments, Caco-2 and HT-29-MTX were seeded together at the same ratio and cell density in 96-well plates (Becton Dickinson).

Cultures were cultivated for 21 days, to reach their full differentiation [11], and the medium was changed every second day. At least 24 hours before the test, the DMEM medium supplemented with penicillin-streptomycin was replaced with the same medium without the antibiotic and the incubation of the cultures was continued. Such prepared cell cultures were used for further experiments, described below.

Adhesion experiment

The Caco-2/HT-29-MTX dimensional co-cultures were incubated with 300 µl of the analysed RH hydrolysate solution at the final concentration of 0.5 µg/ml for two hours at 37°C in an atmosphere of CO₂ and humidity approximately 95%. Cultures without any substrate supplementation were treated as controls (C). After the incubation, the co-cultures were respectively supplemented with 300 µl of het-

erogenous-bacterial isolate solution, prepared as described in the section: bacterial isolates and growth condition. The co-cultures were subjected to a further two-hour incubation. After the incubation, the media were removed from the apical side of the chamber and the co-cultures were washed twice with warm, sterile PBS supplemented with 1 mM of CaCl₂ and 1 mM of MgCl₂ to remove non-adherent bacteria. To liberate the adhered bacteria from the Caco-2/HT-29-MTX surface, the cultures were incubated on ice for 10 minutes with 300 µl of 0.1% cold Triton X-100 solution (Sigma). The liberated bacteria were fixed with 4% PFA and stored at -20°C. Subsequently, bacteria were subjected to DAPI and DOPE-FISH staining procedures, as described below, in order to assess the total number of adhering bacteria and the number of adhering lactobacilli/enterococci (LAB) and enterobacteria (ENT). The experiment was conducted in three parallel repetitions.

Estimation of Total Bacterial Number with DAPI

Shifts in the total bacterial number, caused by exposition to the tested RH hydrolysates, were estimated with DAPI staining according to the procedure described previously by Świątecka *et al.* [12].

Qualitative profile of bacteria with DOPE-FISH technique

Bacterial cells were counted using fluorescent *in situ* hybridisation with double labelling of oligonucleotide probes (DOPE-FISH), according to the protocol described by Rycroft *et al.* [13]. The probes were used as follows: Lab158 specific for *Lactobacillus/Enterococcus* spp. [14] and ENT1432 specific for the bacteria from the Enterobacteriaceae family [15]. The hybridised mixture was subsequently filtered through a 0.2-µm membrane filter (Millipore), and bacterial cells were counted using an epifluorescence microscope (Olympus BX53) and the automatic MultiScan programme for the image analysis. At least 15 random fields were counted on each slide. The analysis was conducted in parallel triplicate.

Physiological response experiment

The co-cultures used in the physiological response experiment are explained on the diagram below (Fig. 2).

A co-culture of Caco-2, HT-29-MTX, and bacteria of different origin was used in order to imitate the small intestinal barrier of healthy and allergic people. Co-cultures of Caco-2 and HT-29-MTX cells, seeded in 96-well plates, were exposed to 100 µl of heterogeneous bacterial solution and incubated for two hours to allow the bacteria to adhere. Co-cultures cultivated without bacteria were treated with 100 µl of DMEM without antibiotics. Afterwards, the non-adherent bacteria were removed by double PBS washing, and subsequently 100 µl of analysed hydrolysates at a final concentration of 0.5 mg/ml were supplemented to

the co-cultures. Hydrolysate solutions were added also to the co-cultures cultivated without bacteria. All co-cultures were incubated for subsequent two hours and subsequently subjected to tests: WST-1, BrdU test, and ELISA method; real-time PCR and TEER (described below).

Additionally, alike cultures were conducted to study the impact of RH hydrolysates on the gene expression. Similar cultures with comparable experimental design were conducted in inserts to assess the impact of RH hydrolysates on transepithelial resistance.

Metabolic activity of Caco-2 cells. WST-1 test

The analyses were carried out after two-hour incubation by adding 10 μ l of the WST-1 reagent (Roche). Samples with the WST-1 reagent were incubated for one hour at 37°C with slight, constant agitation. At the end of the incubation the absorbance was measured at $\lambda = 450$ nm. A sample containing the medium and the WST-1 reagent without any hydrolysate supplementation was treated as a control, whereas a culture containing the growth medium with SDS was a negative control sample. The control sample was treated as 100% and the obtained results were referred to it. The analysis was conducted in parallel triplicate.

Proliferation of Caco-2 cells. BrdU test

To measure DNA synthesis, cells were incubated in the presence of examined hydrolysates at a concentration of 0.5 mg/ml for two hours. After labelling, BrdU incorporation into cellular DNA was measured by colorimetric immunoassay using a commercially available cell proliferation ELISA kit (Roche, France). Absorbance from peroxidase reaction with OPD (o-phenylenediamine dihydrochloride) substrate was measured by a scanning multi-well spectrophotometer at 492 nm. Each experimental condition was conducted in triplicate. BrdU incorporation for all experiments was expressed as the percentage in comparison to the control, which was calculated as 100%.

IL-8 secretion by Caco-2 cells

After the second incubation step, the cell layers were washed two times with pre-wormed PBS. Cytokine production (pg/ml) was compared with the results obtained for cells incubated in the control medium. Each experimental condition was conducted in triplicate. The content of IL-8 in media was measured using a commercially available ELISA according to the manufacturer's instructions (BD Biosciences, OptEIA, Pharmingen, San Diego, CA).

Transepithelial electrical resistance

In order to assess the *in vitro* permeability of the Caco-2/HT-29-MTX cell lines' monolayer, the dimensional culture on hanging inserts was carried out in parallel under the same conditions as described above. At the end of the

experiment, measurement of the monolayer transepithelial electrical resistance was conducted with TEER (Millipore, Merck, Poland). TEER readings were taken at 37°C.

Analysis of mRNA expression of NF κ B and TLR2 genes

Total RNA was isolated from ca. 10⁵ cells stored at -20°C in RNeasy lysis buffer (Qiagen). After defrosting, the cells were washed with sterile, RNase free PBS and proceeded as described in the protocol of the GeneMATRIX Universal RNA Purification kit (Eurobio, Gdansk, Poland). The quality and concentration of isolated RNA was checked spectrophotometrically (PicoDrop, NanoVue). Real-time PCRs were conducted by a MasterGradient Cycler (Eppendorf, Warsaw, Poland) in total volume of 25 μ l. Reaction mixtures consisted of 12.5 μ l of SYBRGreen Jump Start (Sigma), 1 μ l of the reverse transcribed RNA, 1 μ l of each primer, and PCR-grade water (Sigma).

The control was the model of the small intestinal barrier, consisting of Caco-2/HT29MTX/bacteria co-culture.

Statistical analysis

Statistical analysis of the results obtained were conducted with the use of Statistica 9 software. The standard error was used to demonstrate the obtained results. Each mean number of bacteria per cell represents results of three parallel experiments. The statistical significance was determined by the variance analysis using the F distribution by Fisher.

Results

Characteristics of RH hydrolysates

Figure 3 shows the electrophoretic separation of the proteins from rice milk substitute digested by two-step hydrolysis with pepsin/pancreatin.

The proteins from the rice milk substitute were digested in the 70% (Fig. 4).

The impact of hydrolysates on physiological response of Caco-2/HT-29-MTX co-culture with adhered bacteria from healthy people

The presence of the microbial layer, consisting of bacteria obtained from healthy people, altered the response of small intestinal cells when exposed to RH hydrolysates, by increasing the number of proliferating cells and decreasing the secretion of IL-8 in comparison to the co-culture (Fig. 5B.1, 5C.1). However, the observed decrease in the IL-8 secretion had no statistical relevance when compared to the control culture. RH hydrolysates had no statistically relevant impact on the transepithelial transport in the cultures with adhered bacteria from healthy people (Fig. 5D.1).

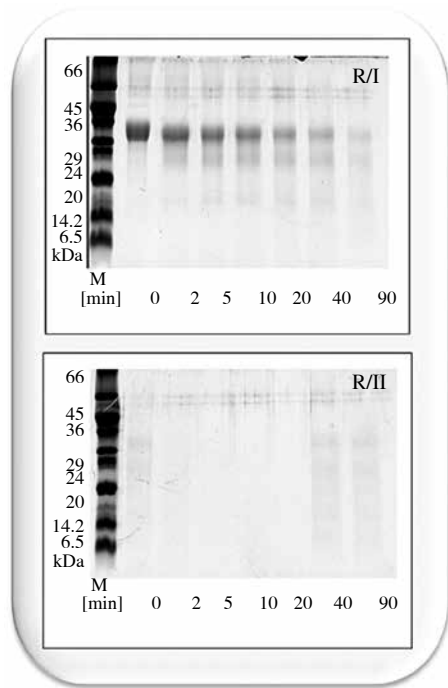


Fig. 3. Kinetics of hydrolysis of proteins from rice milk substitute (R); I – pepsin digestion phase, II – pancreatin digestion phase

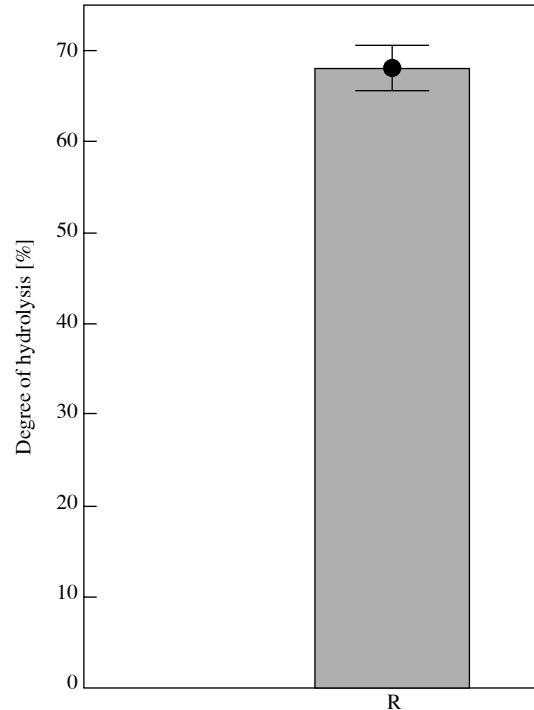


Fig. 4. Degree of hydrolysis proteins from rice milk substitute and RG of proteins from rice milk substitute digested in the presence of glucose

Impact of hydrolysates on physiological response of Caco-2/HT-29-MTX co-culture with adhered bacteria from allergic people

Hydrolysates caused an increase in the metabolic and proliferative activity compared to the control (Fig. 5A, B.2). In addition, the presence of bacteria from allergic people in culture supplemented with RH triggered an increase in IL-8 secretion when compared to the culture with bacteria from healthy people (Fig. 5C.1). RH hydrolysates increased the transepithelial resistance of the epithelial cell layer (Fig. 5D.1).

Analysed hydrolysates had no impact on the total number of adhering bacteria (TBN) in mixed suspension obtained from healthy people (Fig. 6A). Whereas they stimulated the total number of adhering bacteria in mixed suspension obtained from allergic people in the Caco-2/HT-29-MTX co-culture (Fig. 6B). Regardless of the total number of bacteria adhering to small intestinal cells, analysed hydrolysates had no impact on the profile (proportions) of adhering bacteria regardless of their origin, leaving lactobacilli/enterococci more abundantly immobilised in comparison to enterobacteria (Fig. 6A, B).

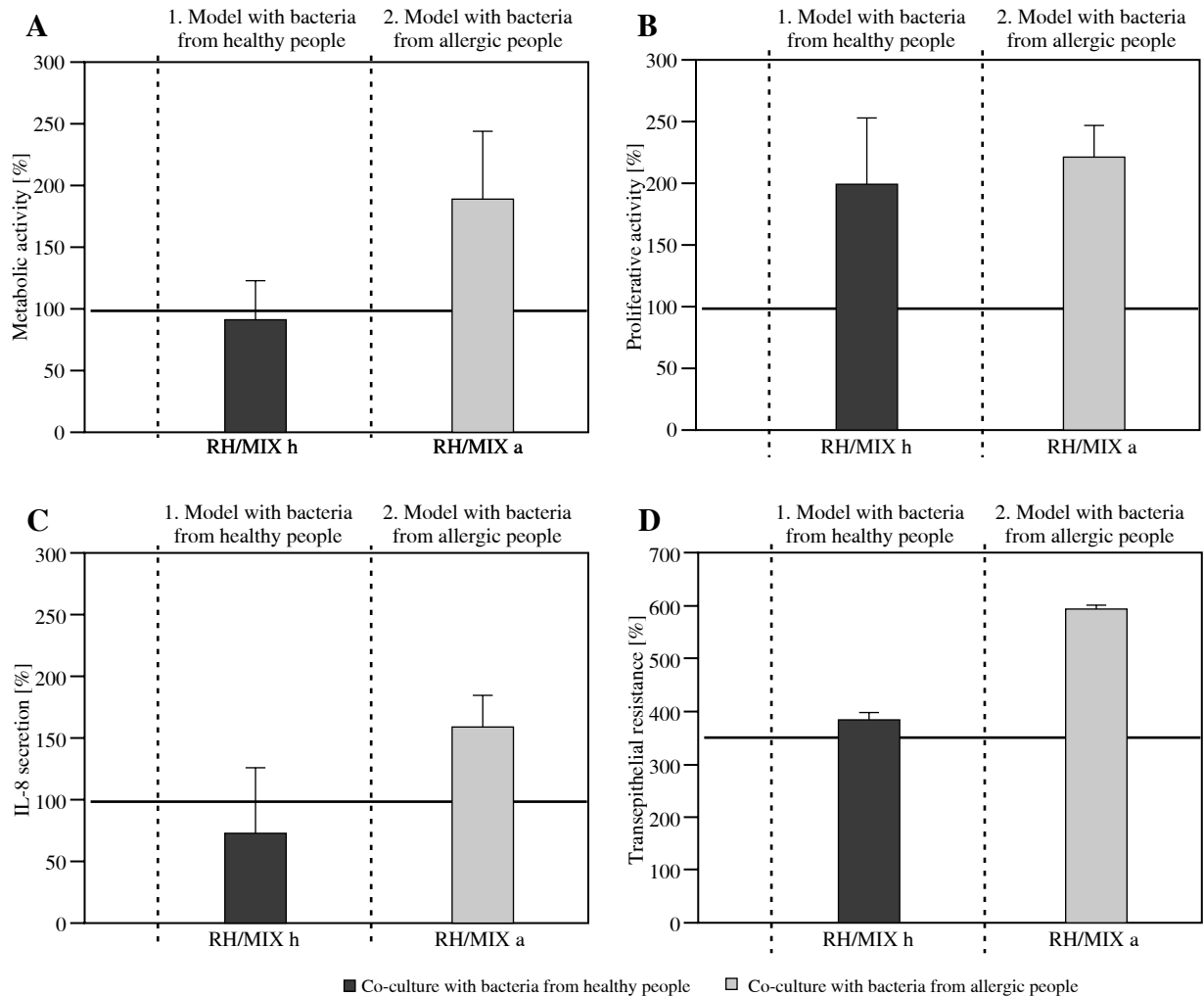
Analysed hydrolysates stimulated the expression of TLR2 gene in the caco-2/HT-29-MTX co-culture with bacteria from healthy people (Fig. 7A).

An opposite effect was exerted by the analysed hydrolysates on the expression of NFκB gene in Caco-2/HT-29-MTX cells with bacteria from various origin, displaying a significant decrease in the cultures with bacteria from healthy people (Fig. 8A) and an increase in the co-culture with bacteria from allergic people (Fig. 8B).

Summarising, the analysed hydrolysate stimulated all of the examined parameters in the intestinal barrier model with bacteria from allergic people, increasing epithelial cells' metabolic and proliferative activity, IL-8 secretion, and transepithelial resistance (Fig. 9B).

Whereas the analysed hydrolysate stimulated solely the epithelial proliferation and transepithelial electric resistance, with no impact on the metabolic activity and IL-8 secretion (Fig. 9) in the intestinal barrier model with bacteria from allergic people (Fig. 9A).

The RH hydrolysates exerted an opposite effect in terms of NFκB expression by epithelial cells, inhibiting it in the co-culture with bacteria from healthy people and stimulating in the co-culture with bacteria from allergic people (Fig. 10). RH hydrolysate stimulated the expression of TLR2 gene in the eukaryotic cells with immobilised bacteria from healthy people and had no impact on its expression in the co-culture with bacteria from allergic people (Fig. 10).



* ($p \leq 0.01$) and ** $p \leq 0.05$. The proper asterisks indicate samples that are statistically different in comparison to the control sample (represented as 100%). The control samples constitute of elements as presented on the scheme below.
 [*] ($p \leq 0.01$) and [**] $p \leq 0.05$. The horizontal line with above asterisks in the brackets indicate samples that differ from each other.

Fig. 5. Impact of hydrolysates of proteins from rice milk substitute (RH) on the physiological response of small intestinal cells (Caco-2/HT-29-MTX) in terms of: A) metabolic activity, B) proliferative activity, C) IL-8 secretion, D) transepithelial resistance

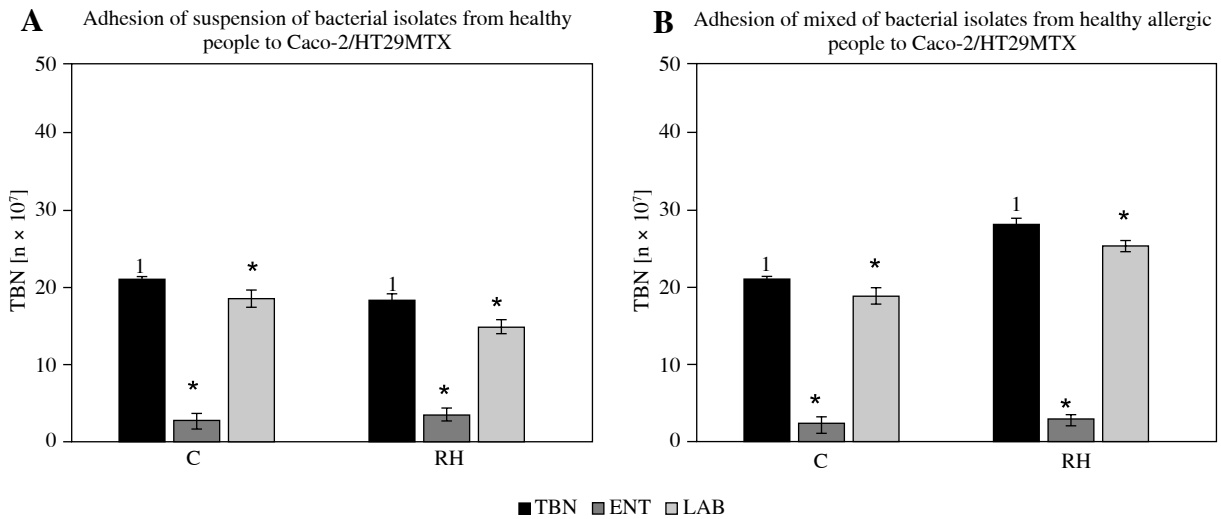
Discussion

Food proteins that are consumed undergo modification due to enzymatic hydrolysis by proteinases present in the GIT, which may lead to the alteration of biological activities displayed by initial protein fractions by the release of highly bioactive peptides and glycopeptides [7]. Enzymatic hydrolysis alters the protein structure, thus modifying its biological activities targeting health effects. It is therefore crucial to take into account the interplay between the small intestinal ecosystem and hydrolysates released by hydroly-

sis in shaping proper physiological condition of the local small intestine environment as well as on the health status of a consumer, while constructing diet recommendations.

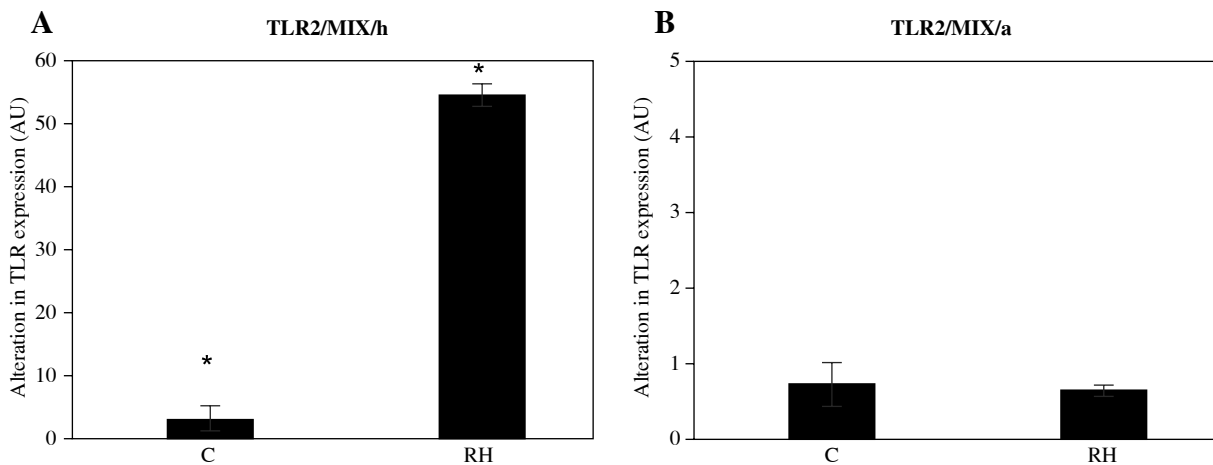
Intestinal barrier model and the impact of hydrolysates on the epithelial response with immobilised bacteria from healthy or allergic people

Reaching the small intestine, the place of final nutrient degradation and absorption, peptides and glycopeptides



* point samples which demonstrate relevant statistical differences ($p \leq 0.01$)
 ENT – Enterobacteriaceae, LAB – *Lactobacillus/Enterococcus*, TBN – total bacterial number

Fig. 6. Impact of hydrolysates of proteins from rice milk substitute – RH on the adhesion of mixed bacterial suspension consisting of Enterobacteriaceae, *Lactobacillus/Enterococcus* to the surface of Caco-2/HT29MTX cells. **A)** Bacteria obtained from healthy people; **B)** Bacteria obtained from allergic people

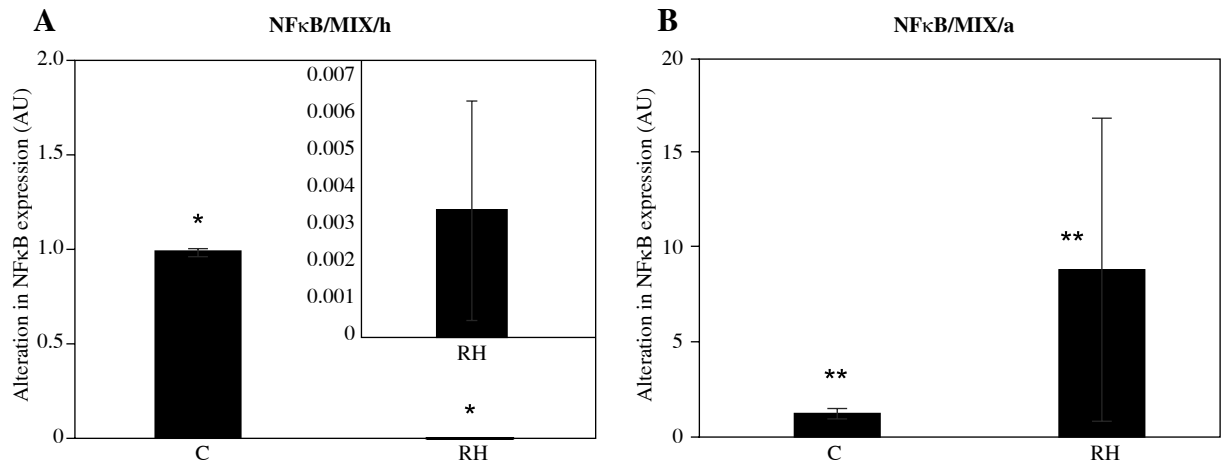


* point samples which demonstrate relevant statistical differences ($p \leq 0.01$).

Fig. 7. Impact of hydrolysates of proteins from rice milk substitute – RH on the expression of TLR2 gene in the Caco-2/HT29MTX cells with adhered bacteria obtained from: **A)** healthy people; **B)** allergic people

act as modulators influencing both the bacteria colonizing this niche, and the eukaryotic cells creating epithelium, which together make up the intestinal barrier. The intestinal barrier is a critical interface between the organism and the environment, guarantying selective permeability, protecting against detrimental factors, and influencing lo-

cal homeostasis [16]. Various *in vitro* models are being used in order to study the physiological status of epithelial cells as well as bacterial adhesion. Most often, such models consist of a single cell line, mainly Caco-2, to imitate the morphology and physiology of the most abundantly present cells: enterocytes. However, the intestinal barri-



* point samples which demonstrate relevant statistical differences ($p \leq 0.01$).

Fig. 8. Impact of hydrolysates of proteins from rice milk substitute – on the expression of NFκB gene in the Caco-2/HT-29-MTX cells with adhered bacteria obtained from: **A)** healthy people, **B)** allergic people

er is a highly complex structure, consisting also of cells secreting mucus and bacteria that are attached to their surface. For that reason, a model consisting of a double cell line culture, encompassing absorptive cells and mucus secreting cell (Caco-2 and HT29-MTX), was applied to assess the biological effect of analysed hydrolysates as well as the mentioned co-culture with bacteria typical for the small intestinal region of different origin (from healthy and allergic people), which were adhered to mentioned epithelial cells. The used cell lines are aimed to retain the phenotypic properties of their progenitors and committed proliferative, differentiated cells to design and maintain the controlled environment. Enterocyte turnover and their optimal physiological status are highly regulated processes to much extend influencing the predisposition to an intestinal inflammation and a number of gastrointestinal diseases, including IBD and food allergy [17].

However, the intestinal barrier consists not only of eukaryotic cells, such as enterocytes and Goblet cells, but also is reinforced by microorganisms attached to their surface [16]. Contact with such immobilised bacteria and/or their products impact the epithelium's turnover, secretive activity, maturation, integrity, and other vital functions [16]. In addition, it impacts the thickness of the intestinal barrier, which is relevant to its physiological function. For microorganisms, adhesion in a mobile environment such as the small intestine, is crucial and enables them to anchor and benefit from the nutritional richness available in this region. Interestingly, when the small intestinal barrier was built up of Caco-2/HT-29-MTX cells and immobilised bacteria obtained from healthy people, the response of epithelial cells to RH hydrolysate shifted significantly

in terms of all of analysed parameters, increasing their mitosis (Fig. 5B.1), and decreasing the IL-8 secretion when compared to the control culture (Fig. 5C.1). That is in accordance with the statement that luminal bacteria signal the interfacing epithelial layer and control the turnover rate of enterocytes, thus fortifying the epithelial regenerative and barrier functions [18]. Consequently, it may positively impact the enterocyte turnover and self-repair, thus having a potentially beneficial effect on the intestinal barrier's status of healthy consumers. The diminished IL-8 secretion may also suppress the immunological response, thus holding back the development of the inflammatory status and barrier impairment. Interestingly, the analysed hydrolysates had no effect on the epithelial integrity in the intestinal barrier model with bacteria from healthy people, thus suggesting the neutral effect of these microorganisms in that aspect (Fig. 5D.1).

A healthy gut remains nonresponsive to a multitude of food antigens and commensal and transient bacteria with an intestinal barrier as a critical factor promoting the differentiation of tolerogenic phenotypes of dendritic subsets, thus implying the importance of local homeostasis for oral tolerance status [19]. The link between disruption in the intestinal barrier functionality and permeability with food allergy is widely accepted, highlighting the importance of epithelial cell integrity, cell shedding, and their activation in terms of chemokine release [20]. A channelled diet-manipulation in terms of consumers suffering from food allergies is therefore crucial in order to avoid triggering the inflammatory status to be more acute and/or to exert health-promoting effects. It is noteworthy that the presence of bacteria isolates from allergic people attached to

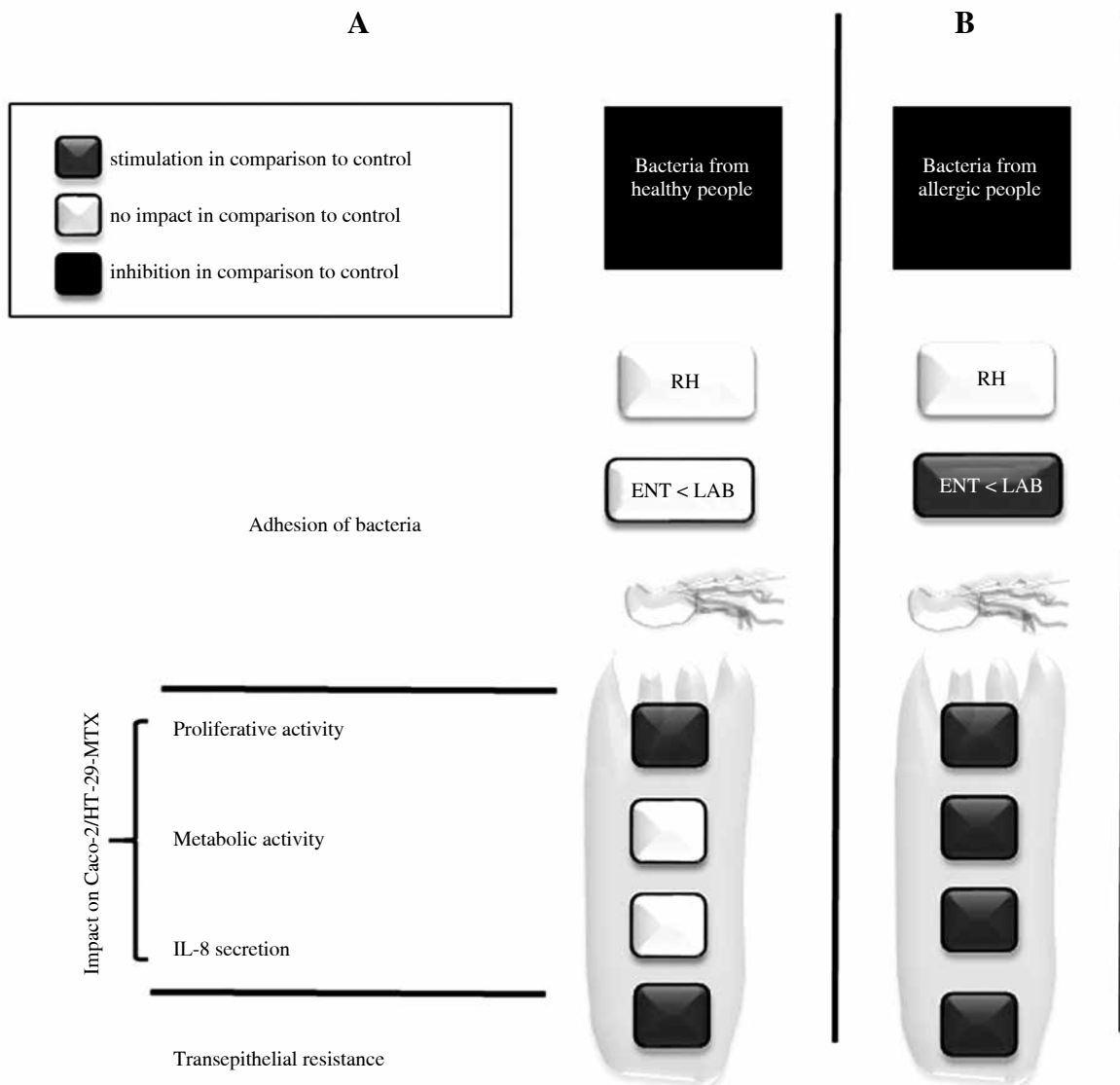


Fig. 9. Schematic summary of impacts exerted by RH hydrolysates of proteins from rice milk on the Caco-2/HT-29-MTX cell lines co-culture and on adhesion of bacteria in the models with bacteria from healthy (A) and allergic people (B)

the epithelial surface along with the presence of examined hydrolysates radically altered the nature of the epithelia's response, triggering the stimulatory effect of proliferation (Fig. 5B.2), metabolic activity (Fig. 5A.), IL-8 secretion (Fig. 5C.2), and integrity (Fig. 5D.2) in comparison to controls without substrate. This response differs from the one observed when bacteria from healthy people constituted the intestinal barrier (Fig. 5.1). Once again, this highlights the importance of cooperation between commensal microbiota and the epithelial cell layer in the aspect of intestinal barrier physiological status. The presence of bacteria from allergic people immobilised to epithelial surface along with RH hydrolysates tunes on the level of proinflamma-

tory cytokine secretion. Such an effect may result in the recruitment of further immune-competent cells in the vicinity of the intestinal mucosa and an ensuing increase of the inflammatory cascade. That may possibly negatively influence the trophic status of the intestinal barriers of people suffering an allergy to milk proteins upon consumption of rice milk or sweetened rice milk. Enterocytes undergo apoptosis and exfoliation as they reach the small intestinal villus tip, and thus, by their shedding, ensuing epithelial renewal and protective function. The increased proliferative activity (Fig. 5B.2) suggests the impact of RH hydrolysates and/or bacteria from allergic people to influence the self-renewal process. However, under inflammatory

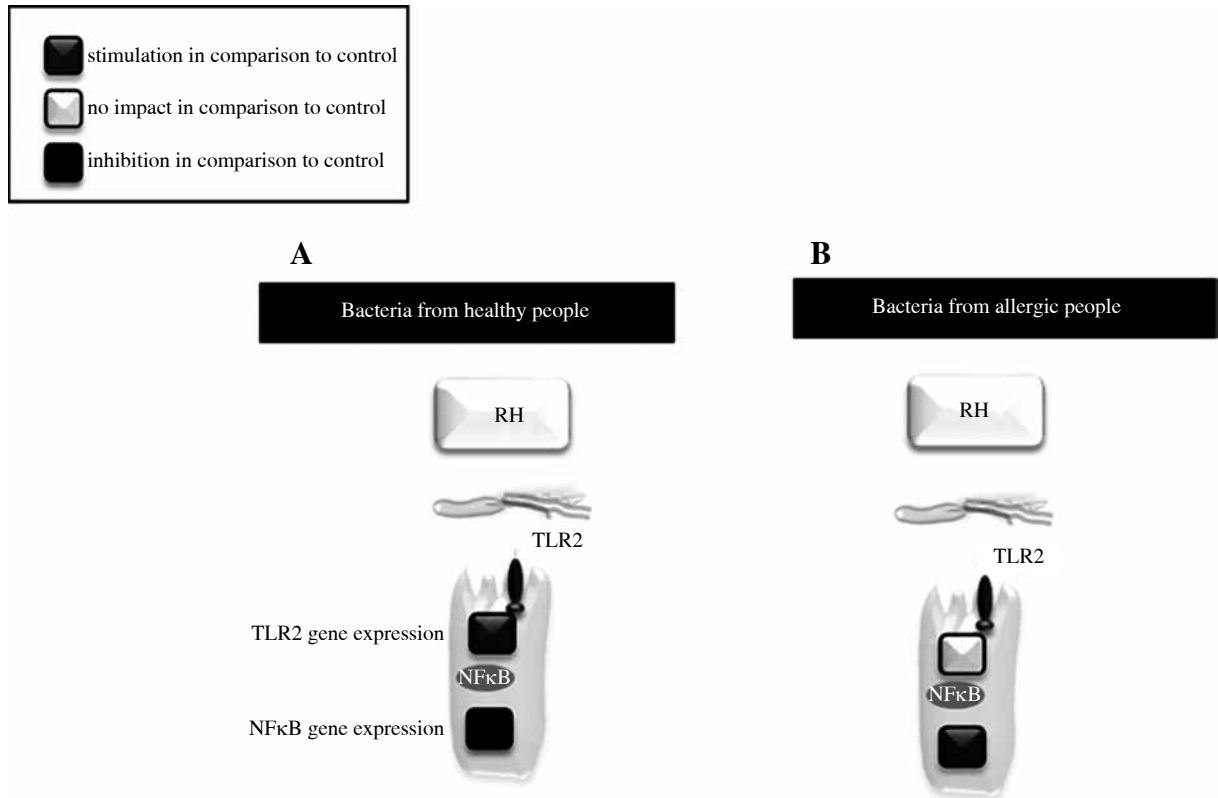


Fig. 10. Schematic summary of impacts exerted by RH hydrolysates of proteins from rice milk substitute on the expression of genes (TLR2 and NFκB) by Caco-2/HT29-MTX cell lines co-cultures with adhered bacteria from healthy (A) and allergic people (B)

conditions, epithelial proliferation and turnover are accelerated, thus increasing the risk of barrier leakage and excessive bacterial translocation, which may induce strong inflammatory responses [21]. On the other hand, being exposed to multiple allergens, epithelial cells are prone to damage, and therefore the increased proliferative activity of epithelial cells along with the proper control of their shedding may be beneficial for epithelial cell self-renewal. This, however, requires *in vivo* verification. The stimulated metabolic activity of epithelial cells suggests their enhanced vital activation, implying their readiness, which may be crucial in terms of fast responses to antigens. The presence of bacteria from allergic people immobilised to the epithelial surface increased its transepithelial resistance in the environment of the analysed hydrolysates (Fig. 5D.2), thus suggesting the increase of the barrier's integrity, which may be a protective response to the local environment shaped by the allergic condition. This implies the possible microbiota-epithelial cooperation determined to protect the human organism. However, a separate study is needed to examine the impact of microbiota from healthy

and allergic people on the physiological status of the intestinal barrier.

The impact of RH hydrolysates on adhesion of bacteria from healthy or allergic people

The environment of the human small intestine is subjected to the constant action of food products, potential allergens, which are decomposed and absorbed in this region, simultaneously influencing bacterial adhesive potential [12]. Adhesion is one of the bacterial strategies indispensable for colonisation of the small intestine [12] and plays a pivotal role in shaping the functionality of small intestinal epithelium, and in influencing the thickness and functionality of the intestinal barrier .

Considered as beneficial, lactic acid bacteria from both healthy and allergic people adhered more abundantly in comparison to enterobacteria, which is a probable result of their adhesive potential determined by specific adhesins accompanied with their ability to compete for receptor sites. Nevertheless, the mechanism of adhesion was not

scrutinised in this study. Such a tendency may result from a mutual interaction between bacteria and eukaryotic cells aimed at optimisation of the intestinal barrier health status; nevertheless, it requires *in vivo* confirmation. The RH hydrolysates had no effect on the profile of adhering bacteria from healthy people, remaining lactobacilli/enterococci most abundantly immobilised to the epithelial surface, as in the control culture (Fig. 6A), implying strong cooperation between prokaryotic and eukaryotic cells. Lactobacilli have been shown to increase tight junction protein expression, consequently restoring intestinal permeability [22]. The fact that the total number of adhering bacteria was not altered by the analysed hydrolysates, nor their profile, may indicate the stability of the intestinal barrier, thus remaining the most abundantly anchored lactic acid bacteria, and that they may potentially exert a beneficial effect by their protective function and metabolic activity [23]. Bacterial adhesion is thought to play a role in the maintenance of the mucus layer by influencing the number and size of mucus secreting Goblet cells [24]. The mucus cover in turn is relevant in terms of trapping unwanted bacteria and thus protecting from the excessive microbe passage through the intestinal barrier.

The impact of the analysed hydrolysates on gene expression

Luminal bacteria are recognised by the extracellularly located part of TLRs, leading to activation of NFκB [25]. Subsequently, activation of NFκB results in the modulation of an inflammatory response. TLR-2 is activated by peptidoglycan, a part of the cell wall of gram-positive bacteria, and its activation leads to phosphorylation of protein kinase C, with a subsequent decrease of intestinal permeability [26]. This decrease in permeability is proposed to be the result of changes in tight junctions. The RH hydrolysates of proteins from rice milk stimulated the expression of TLR2 in epithelial cells co-cultured with bacteria from healthy people (Fig. 7A), implying an increase of the barrier's integrity, potentially beneficial for rice milk consumers. Interestingly, such an epithelial response was not observed in the case of intestinal barrier co-built with bacteria from allergic people. The expression of TLR2 in these cultures was significantly lower when compared to the co-cultures with bacteria from healthy people; this requires further studies to unravel the underlying mechanism. RH hydrolysate had a neutral effect in terms of TLR2 expression.

NFκB is a protein complex causing inflammation in the body by influencing proinflammatory cytokine secretion [20]. Chronic activation of NFκB has been linked to cancer and inflammatory and autoimmune diseases. NFκB is a "rapid-acting" transcription factor, with no requirement of additional proteins for its activation, thus being a first responder to detrimental stimuli. Many bacterial

products and stimulation of a wide variety of cell-surface receptors lead to NFκB activation and fairly rapid changes in gene expression, and a resulting control of low-inflammatory tone and epithelial repair [24]. The beneficial quieting of the NFκB in epithelial cells by both of the analysed hydrolysates was demonstrated in the co-culture with bacteria from healthy people, thus suggesting a beneficial effect. However, the presence of the analysed hydrolysates in the culture with bacteria from allergic people triggered the increase of NFκB expression, thus implying a potentially unfavourable response, especially in the case of allergic people. Since NFκB is a factor involved in the signal transduction via multiple pathways and thus is regulated by various factors, the observed outcome should be scrutinised in a broader manner, taking into account the systemic response in the *in vivo* model.

Conclusions

Bacteria inhabiting the intestinal niche alter the physiological response of epithelial cells to the hydrolysates from rice milk depending on their origin – from healthy or allergic people. With regard to an environment with epithelial cells and bacteria from healthy people adhered to their surface, rice milk protein hydrolysates seem to exert a potentially positive effect by influencing the proliferative activity and decreasing the secretion of proinflammatory IL-8.

However, in terms of the environment consisting of epithelial cells and bacteria from allergic people adhered to their surface, the hydrolysates from rice milk proteins may trigger a potentially detrimental effect due to increased IL-8 secretion and increased mitotic activity, which, under inflammatory conditions, accelerates epithelial proliferation and turnover, thus increasing the risk of barrier leakage and excessive bacterial translocation, which may induce strong inflammatory responses. This, however, requires verification from *in vivo* studies to state diet recommendations.

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