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In vitro evaluation of the effect of the buckwheat protein hydrolysate on bacterial adhesion, physiology and cytokine secretion of Caco-2 cells

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Abstract

Due to the high nutritional value of buckwheat, its protein hydrolysates are a promising candidate for constructing food supplements. However, the preparation of the said hydrolysates requires the analysis estimating their biological activity for the great importance of the gut homeostasis and its impact on the human health status. For that reason, this study aimed at determining the biological function of the buckwheat protein hydrolysates in terms of modulating bacterial activity. The impact of the buckwheat protein hydrolysates on the proliferation and survival rate as well as on the adhesion of bacteria from the following genera: Lactobacillus, Enterococcus, Escherichia as typical representatives of the small intestine microbiota, was studied. In addition, the impact of analyzed hydrolysates on the proliferation of Caco-2 cell and their secretion of interleukin 8 (IL-8) was assessed. The degree of hydrolysis determined the nature of hydrolysates conditioning their size and molecular weight. Although the buckwheat protein hydrolysates slowed down the mitotic process in the intestinal cell and increased the secretion of the proinflammatory cytokine, they also beneficially modulated microorganisms. The buckwheat protein hydrolysates stimulated the proliferation rate of all analyzed bacterial strains with a simultaneous low percentage of dying out. In addition, they facilitated the adhesion of lactic acid bacteria in mono- and heterogeneous cultures and thus are a promising component for compensating the negative response of enterocytic cells.

Key words: *pepsin hydrolysis, bacterial proliferation rate, bacterial survival rate, Caco-2 cell line, bacterial adhesion, cytokine secretion.*

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Introduction

Functional food, tailored for individual humans in order to improve their health status, often consists of highly nutritional valuable proteins and therefore a broad search of such nutrients is constantly conducted. Among plants, buckwheat [*Fagopyrum esculentum* Moench] attracts the attention of consumers, producers, and scientists alike due to its high biological value [1] determined by the abundance of essential amino and fatty acids, vitamins, particularly B1 and B2, and micro- and macroelements [2, 3]. Additionally, the buckwheat grains are known to be an excellent source of rutin, quercetin, kaempferol-3-rutinoside, and a trace quantity of a flavonol triglycoside [4], thus displaying antioxidant, antihemorrhagic and blood vessel protecting properties [5]. The buckwheat grains are also a rich source of fibre, being therefore applied in the prevention of obesity and diabetes [6] and are gluten-free what obliges to use them in the diet composed for people with celiac disease [7].

Protein hydrolysates of plant origin, may be considered as a functional food and good food supplements, mostly due to the fact that being partially digested are a convenient source of valuable amino acids and additionally relieve the gastrointestinal tract [8]. In the light of the high nutritional value, the buckwheat proteins are an attractive source for

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constructing hydrolysates as supplements of human diet. Therefore, it is crucial to analyze the biological activity of the buckwheat protein hydrolysates to ensure that they fulfill consumer expectations. The mentioned biological activity will be strongly linked with the bacterial intestinal ecosystem and enterocytic cells, cooperation of which determines the intestinal homeostasis and in consequence gut health status. Therefore, this study aimed at evaluating the biological function of the buckwheat protein hydrolysates in terms of modulating bacterial activity, expressed as an adhesive, proliferative and survival potential, as well as the physiological response of the intestinal cells determined by the shift in their proliferation rate and cytokine secretion.

Material and methods

Material

The raw buckwheat seeds (*Fagopyrum esculentum* Moench, KORA variety) were delivered from the Production and Experimental Research Station, located in Bałcyny, Poland, and were subsequently ground to flour in a WŻ-1 grinder (ZBPP Sadkiewicz Instruments, Poland) and later on sieved through a standard 0.20 mm sieve (NAGEMA, Germany).

Protein extraction and determination of protein contents

Samples (35 g) of the ground pea flour were extracted with 140 ml of 50 mM Tris-HCl (pH 8.8) for 1 hour at 4˚C with constant agitation and subsequently centrifuged (20 000 g, 20 min). The extraction was repeated twice. The supernatant, containing albumins and globulins, was dialysed at 4˚C for 48 hours against distilled water and later lyophilised [9, 10]. Concentrations of proteins were determined using the Bradford's method [11] with bovine serum albumin (BSA) as a standard.

Preparation of the buckwheat proteins digests

The kinetic analysis of the samples was carried out at pH 2.0 for pepsin hydrolysis (one-step hydrolysis). Three suspensions of 0.6 g of the buckwheat protein extract in 200 ml of distilled water at pH 2.0 (obtained by adding 1 M HCl) were prepared. Each of the prepared suspensions was incubated with 5.81 mg, 11.62 mg and 17.43 mg of pepsin (Sigma P-7000) for 2 hours at 37˚C with constant agitation. The hydrolysis was stopped by freezing at –20˚C.

Determination of the degree of hydrolysis

Total contents of α-amine groups (h_{total}) were determined according to the method reported by Hajos *et al*. [12]. The solution of 10 ml of 6 M HCl and 0.5 g of the buckwheat protein extract was put in a glass ampoule and saturated with nitrogen. Next, the ampoule was closed by heating. The hydrolysis was conducted at 105˚C for 12 h. The solution was filtrated and neutralized with 6 M NaOH, and subsequently transferred to a volumetric flask and adjusted with phosphate buffer (pH 8.2) to its final volume of 100 ml. The content of α-amine groups was determined with the OPA method. The degree of hydrolysis (DH) was calculated using the following equation [13]:

$$DH = h/h_{total} \times 100\%$$

where:

h – number of hydrolyzed peptide bonds (mEq Leu – NH_2/g of protein),

h_{total} – total number of peptide bonds in the protein (mEq Leu – NH_2/g of protein).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the buckwheat proteins and buckwheat protein hydrolysates was carried out using a discontinuous system according to Laemmli [14] with 4% stacking and 15% separating gel for the buckwheat proteins and 4% stacking and 20% separating gels for the buckwheat protein hydrolysates using the apparatus Medium (Kucharczyk Co., Poland). The gel with the buckwheat proteins was run in 0.1 M Tris-glycine buffer at pH 8.3, whereas the gels with the buckwheat protein hydrolysates were run in two buffer systems: an upper one – Tris-tricyne, pH 8.3 and a lower one – Tris-HCl, pH 7.5. The gels were stained with Coomassie Brilliant Blue R-250 (Serva Stain Biochemica GmbH, Heidelberg, Germany). A low molecular weight markers kit (6.5–66.0 kDa) by Sigma (M-3913) was used to estimate the band size.

Bacterial strains and growth conditions

Bacterial strains were chosen on the basis of the results obtained from the screening disc diffusion method using various microorganisms. The selected strains are representatives of microbes typical of the human small intestine. The strains: *Enterococcus faecium* PCM 1859, *Escherichia coli* PCM 360 were obtained from the Polish Collection of Microorganisms, the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences (Wroclaw, Poland), whereas *Lactobacillus acidophilus* 15 was obtained from the Faculty of Food Science of the University of Warmia and Mazury (Olsztyn, Poland). The strains were isolated from human faeces.

Cultivation of these bacteria was carried out aerobically at 37˚C. *Escherichia coli* was grown on the nutrient broth medium (Biocorp), *E. faecium* was grown on the Brain Heart Infusion medium (BHI, BioMerieux), whereas *L. acidophilus* was cultivated on the MRS medium (BTL, Poland).

Preparation of the bacterial inoculum

Bacteria were transferred from agar slants into appropriate liquid medium and incubated for 24 hours at 37˚C.

The overnight cultures were refreshed by transferring 0.5 ml into freshly prepared liquid media (10 ml) and again incubated under identical conditions with constant absorbance measurement until reaching A_λ = 550 nm = 0.2. Such prepared bacterial suspensions were used for inoculation in further microbial analysis.

Methods used in microbial analysis

Estimation of the total bacterial number with 4',6-diamidino-phenylindole

The total bacterial numbers resulting from the exposition to the tested buckwheat proteins and their hydrolysates, were estimated with the use of staining with 4',6-diamidino-phenylindole (DAPI) [15]. Bacterial samples fixated with formalin (final concentration 4%) were stained with DAPI for 15 minutes at room temperature, in the dark and afterwards filtrated trough 0.2 µm black polycarbonate filters (Millipore), air-dried and mounted on a microscopic slide with a drop of Citifluor (Cargille). Next, they were analysed using an epifluorescence microscope (Olympus U-RFL-T) equipped with the following filters: BP 360 nm, BA 420 nm, DM 400 nm and the automatic MultiScan programme for image analysis. At least 15 random fields were counted on each slide. The analysis was conducted in parallel triplicate.

Estimation of the bacterial survival rate with Live/Dead Bacterial Viability Kit

The samples were collected and incubated with the Live/Dead Bacterial Viability Kit (Molecular Probes) to determine the percentage of dead bacterial cells [16]. After 15 minutes of incubation with the Kit, the samples were filtered through 0.2 µm black polycarbonate filters (Millipore), air dried and mounted on microscopic slides with a drop of BacLight Mounting Oil (Molecular Probes) and subsequently analysed microscopically. The filters BP 530-550 nm, BA 590 nm, DM 570 nm were used to determine the percentage of dead bacterial cells. The analysis was conducted in parallel triplicate.

Estimation of the bacterial cell number with fluorescent *in situ* hybridization

The bacteria were counted using the fluorescent *in situ* hybridization (FISH), as described by Rycroft *et al*. [17]. The samples were diluted four times in 4% (w/v) filtered paraformaldehyde and fixed overnight at 4°C. Afterwards, the samples were washed twice with filtered PBS and stored at –20°C in PBS/ethanol solution (1 : 1, v/v) until further analysis. The hybridisation was conducted at the appropriate temperature using genus-specific 16S rRNA-targeted oligonucleotide probes labelled with the fluorescent Cy3 dye for different bacterial groups or with 4',6-diamidino-2-phenylindole (DAPI) for total cell counts. The probes used were: Lab 158 specific to *Lactobacillus/Enterococcus* spp., EC 1531 specific to *E. coli* [18]. The hybridised mixture was subsequently filtered using a 0.2-µm membrane filter (Millipore) and the bacterial cells were counted using an epifluorescence microscope (Olympus U-RFL-T) equipped with the MultiScan Programme for image analysis. At least 15 random fields were counted on each slide. The analysis was conducted in triplicate.

Experimental approach

Bacterial proliferation rate in the liquid cultures

Liquid, 100-fold diluted media for bacterial growth (as described in the section "Bacterial Strains and Growth Conditions") were supplemented with the solution of the buckwheat protein hydrolysate to achieve the final concentration of 1 mg/ml (calculated as 1 mg of protein/ml). Cultures without any buckwheat protein hydrolysate supplementation were treated as controls of the bacterial growth rate. Such prepared cultures were inoculated with 1% of a particular bacterial strain and incubated at 37°C with constant, gentle agitation for optimal nutrients accessibility. Four, experimentally estimated, measurements were conducted during the cultivation period to study the shift in the total bacterial number with DAPI (according to the procedure described above).

Bacterial survival rate in the liquid cultures

The experiment was conducted similarly as the one described above. The exception was that the samples were taken to examine the bacterial survival rate by assessing bacterial membrane integrity with Live/Dead (according to the procedure described above).

Caco-2 cell line

The Caco-2 cell line was obtained from the American Type Cultures Collection. The Caco-2 cells were routinely cultured 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 acid mixture (NEAA, Gibco) and 50 mg/ml of gentamycin (Gibco). The incubation was carried out at 37°C in 5% of CO_2 atmosphere and the humidity of approximately 95%. 21-day-old Caco-2 cultures were used in the experiments as differentiated and fully confluent cells according to Peterson and Mooseker [19]. Caco-2 cell lines were used to assess the impact of the buckwheat hydrolysates on the adhesion of mono- and heterogeneous bacterial cultures and physiological response of enterocytes (cytokine secretion and proliferation).

Bacterial adhesion experiments

The Caco-2 cells were seeded at the concentration of 2.5×10^4 cells/well in 96-well standard tissue culture plates (Becton Dickinson). At least 24 h before the test, the DMEM medium supplemented with gentamycin was

replaced with the same medium without the antibiotic and the incubation of the cultures was continued. Such prepared cultures were used for further studies described below.

Adhesion assay

The assay was conducted according to the procedure of Świątecka *et al*. [20]. The Caco-2 monolayer was incubated for 2 h at 37°C in the atmosphere described previously with 50 µl of the buckwheat proteins and the analysed buckwheat protein hydrolysate at the final concentration of 1 mg/ml. After the incubation, the cultures were respectively supplemented with 100 µl of an inoculating solution containing 25% of the particular bacterial inoculum prepared as described above, and 75% of the DMEM medium without gentamycin. The cultures were subjected to a further 2-hour incubation. The cultures without any buckwheat proteins nor the hydrolysate supplementation were treated as controls (C). After the incubation, the media were removed and the cultures were washed out twice with warm, sterile PBS to remove non-adherent bacteria. To liberate the adhered bacteria from the Caco-2 surface, the cultures were incubated on ice for 10 minutes with 200 µl of 0.1% ice-cold Triton X-100 solution (Sigma). The solutions containing the bacteria were stained with DAPI in the procedure described above in order to assess the number of bacteria attached to the Caco-2 surface. The analysis was conducted in three parallel repetitions.

Competition adhesion test

Heterogeneous culture, consisting of commensal bacteria from genus *Escherichia* and beneficial lactic acid fermenting bacteria from genera *Lactobacillus* and *Enterococcus*, was used in the study in order to assess the microbial competition in the adhesion to the surface of Caco-2 cells. The examined buckwheat protein hydrolysate solution was added to the cultures in the volume of 50 µl and at the final concentration of 1 mg/ml. The cultures were subsequently incubated for 2 h at 37°C. At the end of the incubation period, the inoculation solution, in the volume of 100 µl, was added to the cultures and it continued to be incubated for 2 h. The inoculation solutions consisted of 40% *L. acidophilus*, 30% *E. coli* and 30% *E. faecium*. The cultures without any buckwheat protein hydrolysate supplementation were treated as controls (C). After the incubation, the media were removed and the cultures were washed out twice with warm, sterile PBS to remove non-adherent bacteria. To liberate the adhered bacteria from the Caco-2 surface, the cultures were incubated on ice for 10 minutes with 200 µl of the 0.1% cold Triton X-100 solution (Sigma). The solutions containing bacteria were hybridised with EC 1531 and LAB 158 probes in order to estimate the cell number of particular bacteria (according to the procedure described above).

Impact on the cytokine secretion determined with enzyme linked immunosorbent assay (ELISA)

Post-confluent Caco-2 cells were washed two times with pre-warmed PBS. To measure IL-8 secretion cells were incubated in the presence of the buckwheat protein hydrolysate at a concentration of 1 mg/ml for 2 hours. Incubation time and protein hydrolysates concentration were estimated and optimized in previous studies. Cytokine production was compared with the results obtained for cells incubated in the medium without any supplementation. Each experimental condition was analysed in parallel 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).

BrdU incorporation

Caco-2 cells were plated in 96-well plates and cultured as described above. To measure DNA synthesis, cells were incubated in the presence of the buckwheat protein hydrolysates at a concentration of 1 mg/ml during 2 hours. After labelling, BrdU incorporation into cellular DNA was measured by a colorimetric immunoassay using a commercially available cell proliferation ELISA kit (Roche, France). Absorbance from peroxidase reaction with OPD (o-Phenylenediaminedihydrochloride) substrate was measured by a scanning multi-well spectrophotometer at 492 nm. Each experimental condition was conducted in quadruplicate. BrdU incorporation for all experiments was expressed as the percentage of proliferation in relation to the control value (100%) obtained for cells incubated with pure medium.

Statistical analysis

The Statistica 9 programme was used to conduct statistical analysis of the results obtained. The standard error was used to demonstrate the obtained results. Each mean number of bacteria per cell represents results of three experiments. The statistical significance was determined by the variance analysis using the F distribution by Fisher.

Results

Figure 1A shows the electrophoretic separation of the buckwheat proteins (BP). The electropherogram displayed 18 bands representing molecular weights ranging from 7.6 to 82.0 kDa. Buckwheat protein consists of 18.2% of albumin, 43.3% of globulin, 0.8% of prolamin, 22.7% of glutelin and 5.0% of other nitrogen residue [21]. As reported by Javornik *et al*. [22] the buckwheat salt-soluble globulin is mainly represented by the 13S 280 kDa component, which consists of three fractions with molecular masses between 43-68, 57-58, 26-36 kDa. Fractions of 8-16 kDa are considered to form 2S albumin [23]. The 8S globulin is con-

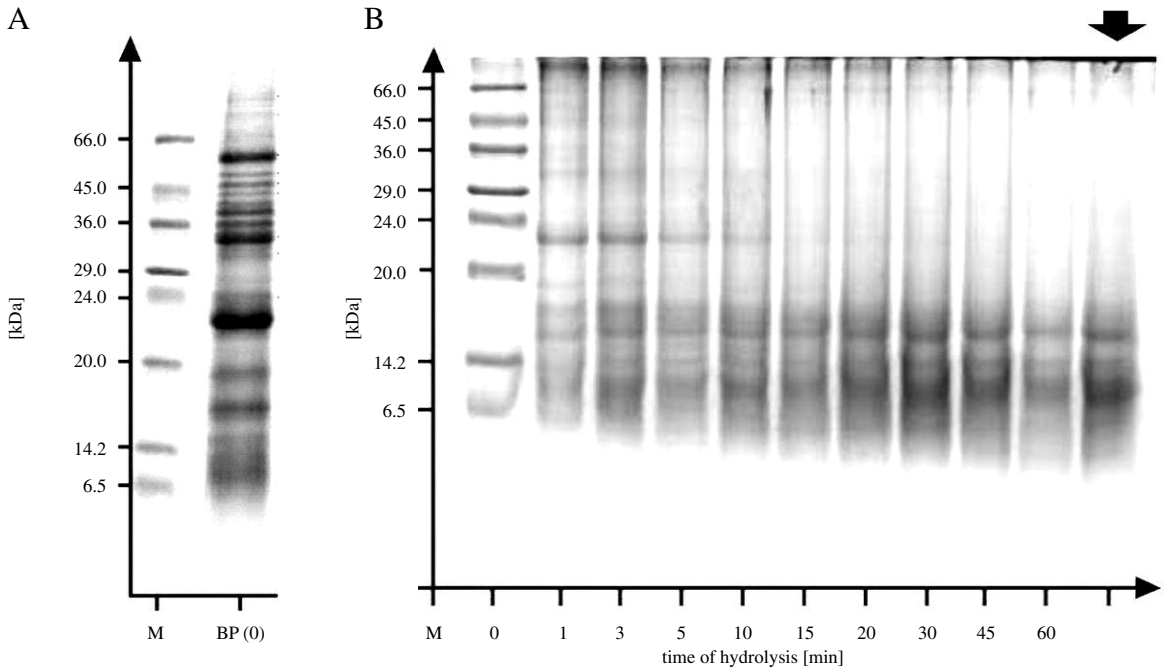

Fig. 1. Electropherogram of the buckwheat proteins (A) and the buckwheat protein hydrolysates – kinetics of the hydrolysis process (B). Legend: M – molecular weight marker, BP – buckwheat proteins; A) electropherograms ran in 15% separating gels, B) electropherograms ran in 20% separating gels. An arrow indicates the buckwheat protein hydrolysate that was used in the study.

sidered to be a trimer composed of fractions with molecular masses between 57 to 58 kDa [23]. The major prolamin bands according to Guo and Yao [24] are 20 and 14 kDa. The hydrolysis process was carried out dynamically, after 10 minutes degrading the high molecular weight protein fractions. After 60 minutes the buckwheat proteins of high molecular weight were hydrolysed remaining fractions of the weight below 20 kDa (Fig. 1B).

The kinetics of the hydrolysis of the buckwheat proteins was expressed as a function of the change in the degree hydrolysis (DH) in time and is presented in Fig. 2. The hydrolysis process was very rapid during the first 5 minutes and triggered the shift in the protein profile, causing the decrease in the main protein fraction with a subsequent increase in the polypeptide and peptide fractions. Enzymatic activity of pepsin on the buckwheat proteins led to their approximately 5% hydrolysis at 60 min.

The stimulatory impact of the buckwheat protein hydrolysates on the proliferation rate of *E. coli*, *E. faecium* and *L. acidophilus* is presented in Fig. 3. The observed effect did not depend on the genus nor the bacterial cell wall type (gram positive and negative bacteria). Moreover, the supplementation of cultures with the buckwheat protein hydrolysates maintained the cultures in good condition with a low percentage of dead cells in comparison to the control culture (3-20%; Fig. 4).

Figure 5 demonstrates the impact of the buckwheat protein hydrolysate on the adhesion of particular bacteria to the

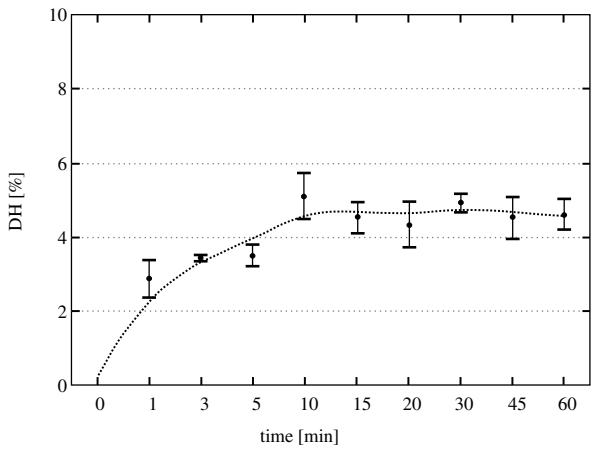

Fig. 2. Degree of hydrolysis (DH) of the buckwheat proteins hydrolyzed with pepsin

surface of Caco-2 cells. The hydrolysate of the buckwheat proteins showed no impact on the adhesion of *E. coli* and enterococci in comparison to the control cultures (Fig. 5A, B, respectively). Interestingly, it stimulated the adhesion of beneficial lactobacilli by 100% ($P < 0.05$?).

The buckwheat protein hydrolysate stimulated the adhesion bacteria expressed as a total cell number in comparison to the control culture although with no statistical relevance (Fig. 6). However, the buckwheat protein hydrolysate

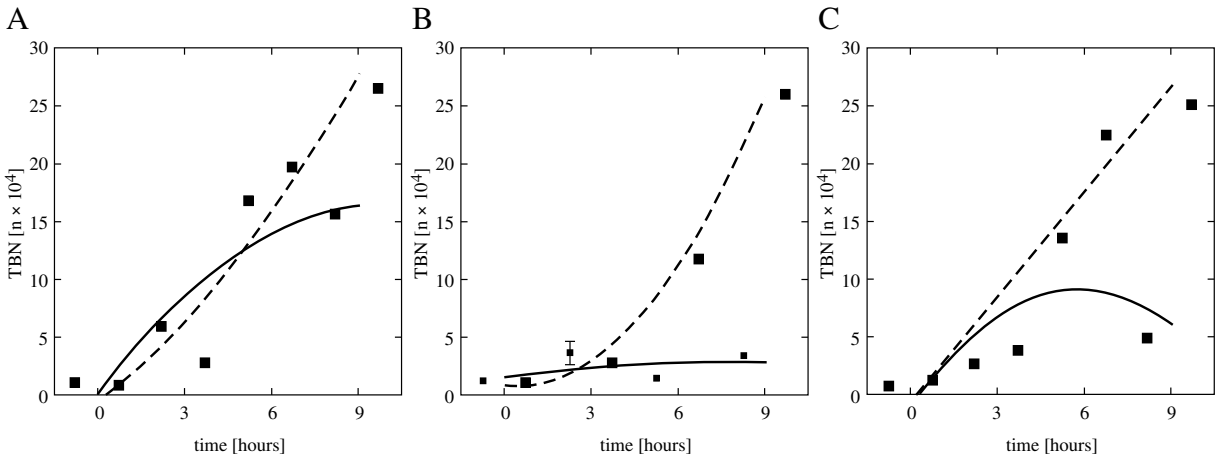

Fig. 3. The proliferation rate of: A – *E. coli*, B – *E. faecium*, C – *L. acidophilus*. TBN – total bacterial number, the control culture without any hydrolysate supplementation, the culture supplemented with the buckwheat proteins hydrolysate

significantly altered the qualitative ratio of immobilized bacteria with a higher number of beneficial lactobacilli and enterococci attached to intestinal cells.

The buckwheat protein hydrolysate triggered the drastic decrease in an actively proliferating intestinal cells in comparison to the control culture ($P < 0.01$?; Fig. 7). It implies that that hydrolysate hampered the mitotic proliferative process of the Caco-2 cells.

When exposed to the buckwheat protein hydrolysate Caco-2 cells secreted a significantly ($P < 0.01$?), 8-fold higher, amount of proinflammatory cytokine IL-8 in comparison to the control culture (Fig. 8).

Discussion

Although enzymatic hydrolysis of food proteins is a substantial process frequently used to improve the physical, chemical, functional and nutritional properties of native proteins [25], its complexity requires selection of optimal conditions. The enzymatic reaction in this study was carried out under optimal conditions created to imitate the ones occurring *in vivo.* When subjected to SDS-PAGE, the buckwheat protein hydrolysates demonstrated a decreasing number of fractions, particularly the ones of high molecular weight (Fig. 1B). The cause of observed phenomena was structural alterations ensuing from the enzyme-substrate interaction leading to unfolding of the buckwheat proteins and thus enabling pepsin to release shorter-chain fractions. Nutrients reaching the lower parts of the gastrointestinal tract are mainly subjected to the enzymatic degradation performed by microbes, producing a variety of proteinase activities [26] and thus modulating their activity and quantitative and qualitative parameters. For that reason, evaluation of the impact of the buckwheat protein hydrolysates, as potential food supplements, is of great importance in terms of intimate understanding of their biological activity as well as maintenance of the gut homeostasis and ensuing the good health status of a consumer.

The cell wall-type independent stimulatory effect of the buckwheat protein hydrolysates implies their potential usage as a good energy and nutrients source (Fig. 3). The strong stimulatory effect observed maintained the growth of bacteria from the genera *Escherichia* (Fig. 3A), *Enterococcus* (Fig. 3B) and *Lactobacillus* (Fig. 3C) in the log phase of growth. The preferential utilization of these substrates by mentioned bacteria may act as a mutual effect resulting from the nature of peptide fractions themselves as well as bacterial enzymatic equipment and their absorptive abilities. It is noteworthy that the proteinase activity is strongly associated with the bacterial groups [27]. Moreover, microorganisms have developed a wide spectrum of mechanisms controlling the proteinase activities, including various levels of their regulation [28]. The increased proliferation rate of *E. coli*, *E. faecium* and *L. acidophilus* cultures supplemented with analyzed substrates determined their good physiological condition with a relatively low percentage of dead bacterial cells in comparison to the control cultures (Fig. 4). It proves that examined substrates were used for anabolic pathways simultaneously hindering the lysis process. Similar observations were presented in the study concerning the pea protein hydrolysates conducted by Świątecka *et al.* [29]. The surprisingly high percentage of the dead lactobacilli cells at the onset of the cultivation period gives evidence to the adaptive requirements of these microorganisms to brand new environmental conditions. The presence of the mentioned substrates was a probable

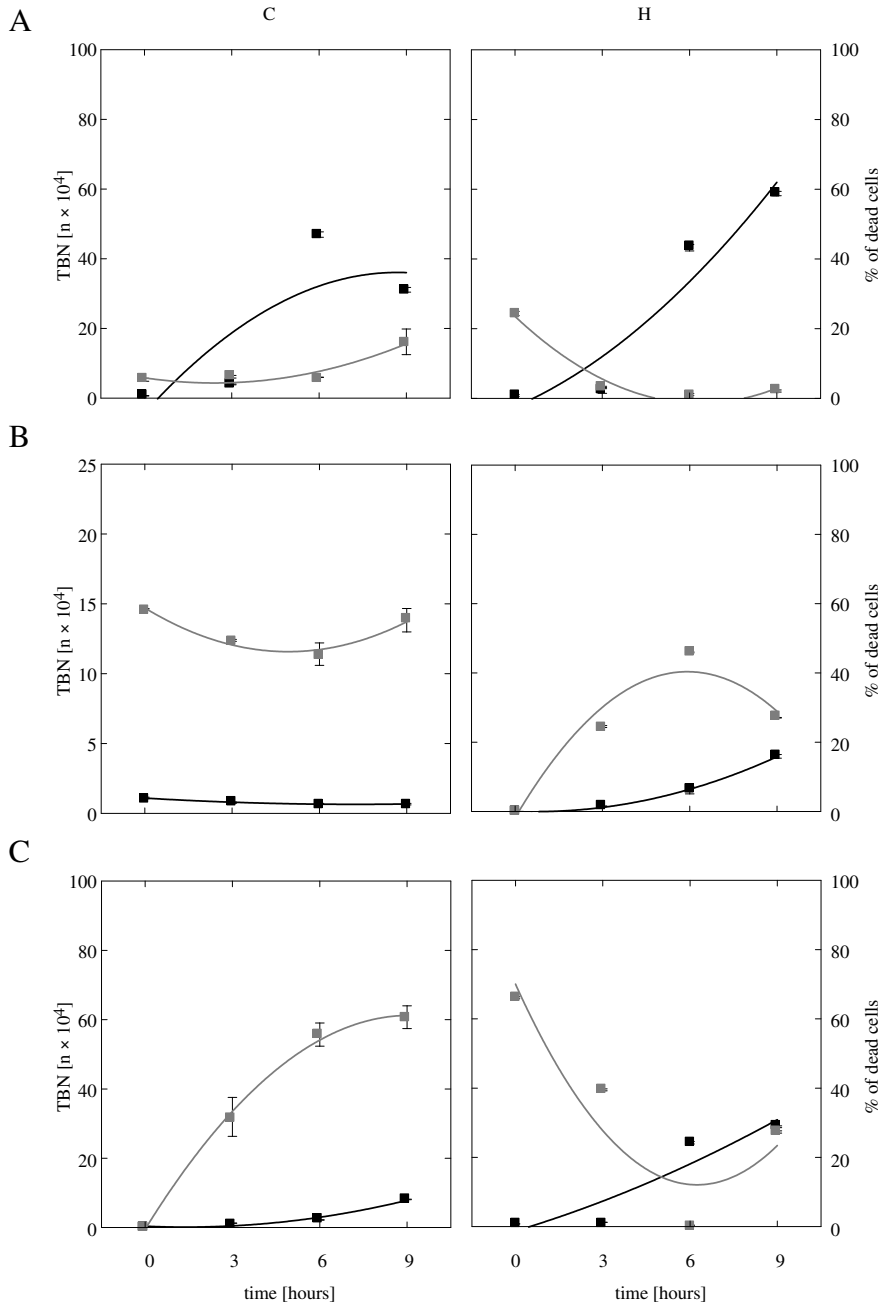

Fig. 4. The survival of bacteria expressed as a total bacterial number (TBN). Vertically: A – *E. coli*, B – *E. faecium*, C – *L. acidophilus*. Horizontally: C – the control culture without any hydrolysate supplementation, H – the culture supplemented with the buckwheat protein hydrolysate, ■ TBN, ■ % of dead bacterial cells

factor altering the pH and the ionic strength of the medium, which are considered to be factors controlling activities of proteases [28]. The subsequent decrease in the percentage of dead lactobacilli implies that these bacteria had adopted to the new environment and allegedly had activated proper enzymatic systems tailored to utilize available substrates and to absorb them. The supplementation of medium with protein hydrolysates may promote the acidification of the local environment and by such means increase the survival rate of lactobacilli, what was demonstrated in the study of Amiot *et al*. [30].

The bacterial adhesion to the surfaces of enterocytes not only determines their ability to stay in the mobile environment of the small intestine and their access to nutrients but

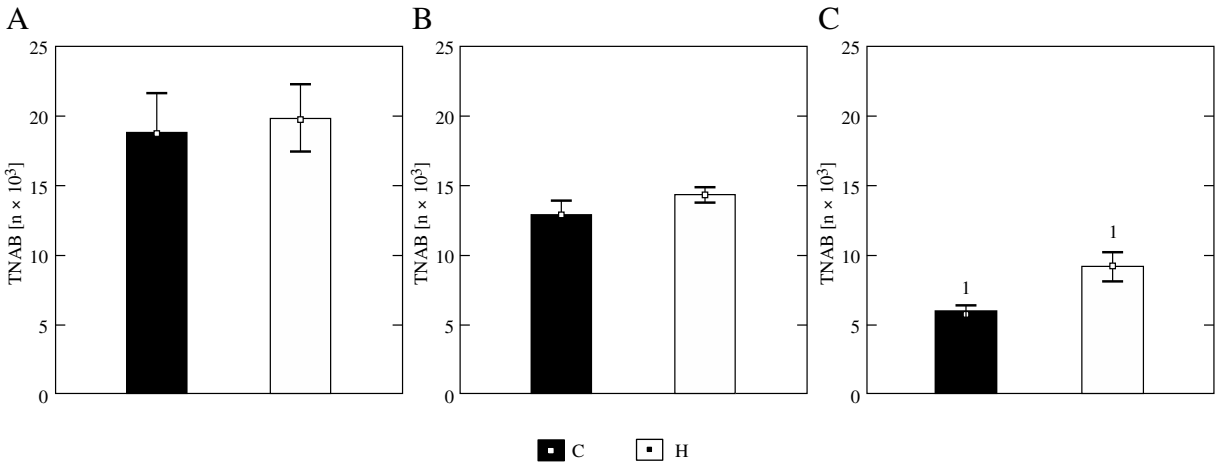

Fig. 5. The adhesion of: A) *E. coli*, B) *E. faecium*, C) *L. acidophilus* to the surface of Caco-2 cells exposed to the buckwheat protein hydrolysate. Legend: C – the culture without any hydrolysate supplementation, H – the culture supplemented with the buckwheat protein hydrolysate. Identical Arabic numerals point out to samples demonstrating relevant statistical differences ($P < 0.05$)

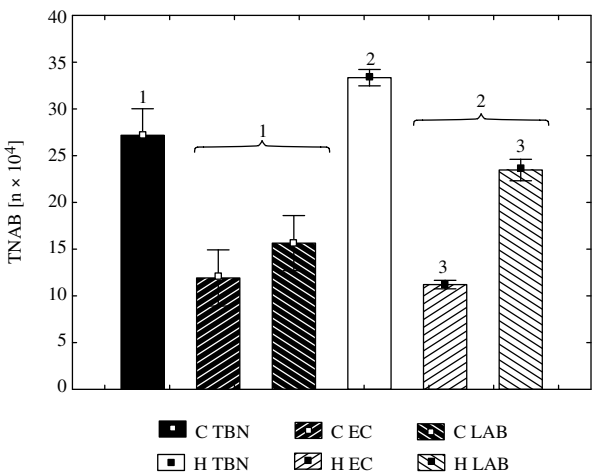

Fig. 6. The competition in adhesion between *Lactobacillus/Enterococcus* and *Escherichia* to the surface of the Caco-2 cells in the environment of the analyzed hydrolysate. Legend: C – culture without any hydrolysate supplementation, H – culture supplemented with the buckwheat protein hydrolysates; TNAB – Total Number of Adhering Bacteria, LAB – number of adhering lactobacilli/enterococci, EC – number of adhering *E. coli.* Identical Arabic numerals point out to samples demonstrating relevant statistical differences at $P < 0.05$

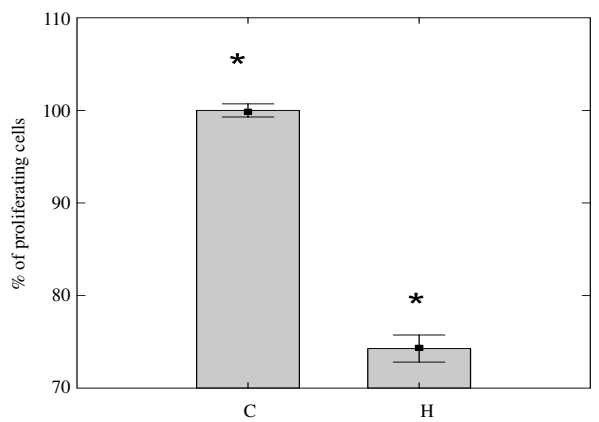

Fig. 7. Proliferation of Caco-2 cells exposed to the buckwheat protein hydrolysate (H). C – control culture without any protein hydrolysates supplementation. Cultures demonstrate statistical differences at $P < 0.01$

also brings about a benefit to the human organism by creating and maintaining the barrier protecting from the invasion of pathogens, influencing the epithelial physiological activity, enhancing healing of a damaged mucosa as well as stimulating the immunological system [31]. The preclusion of the evaluation of the bacterial adhesion *in vivo* necessitates the usage of appropriate *in vitro* models. The cell lines used as such models are aimed to retain the phenotypic properties of their progenitors and committed proliferative, differentiated cells to design and maintain the controlled environment [32]. Hence, in spite of the fact that the Caco-2 cell line was originally obtained from a human colon adenocarcinoma, it acquires many small intestinal cell phenotypes, such as levels of alkaline phosphatase, sucrase-isomaltase, and aminopeptidase activities similar to those of the small intestine enterocytes [33]. The mentioned properties justify using the Caco-2 cell line as a model system to study the bacterial adhesion [34]. The hydrolysate analyzed in this study had no influence on the adhesion of bacteria from genera *Escherichia* and *Enterococcus* (Fig. 5A, B),

what suggests that despite being a favorable nutrient and energy source, these hydrolysates do not act as a linkage between bacterial and eukaryotic cells guaranteeing their adherence. In this case, adhesion seems to be strain-dependant and is likely to be determined by the presence of adhesins on respective bacterial surfaces and their affinity to receptors expressed on the epithelium, not by the bacterial competitors for the adhesive sites on epithelium increased due to the stimulatory effect of the analyzed hydrolysate in terms of the proliferation rate. Interestingly, the buckwheat protein hydrolysate stimulated the immobilization of lactobacilli to the intestinal surfaces (Fig. 5C). Substrates stimulated the lactobacilli's growth, thus increasing the number of microbes that might have strived for adherence and such a change might be correlated with the increase in bacterial immobilization, as it was in the case of *L. acidophilus* (Fig. 5C).

The intestinal ecosystem is a habitat of heterogeneous bacterial populations and therefore the impact of the buckwheat protein hydrolysate on the adhesion of mixed cultures of *E. coli*, *E. faecium* and *L. acidophilus* (Fig. 6) was studied. The mentioned experiment involved bacteria from genera stated above as typical representatives of microorganisms colonizing the human small intestine. The supplementation of cultures with the buckwheat protein hydrolysates enhanced the adhesion of total bacteria when compared to the control with a simultaneous increase in immobilized lactic acid bacteria thus indicating their beneficial biological effect (Fig. 6). Although Lee and Puong [35] reported that the adhesion of lactobacilli occurs mostly via hydrophobic interactions that influence the competition for a specific receptor, these bacteria are also known to be equipped with surface adhesins that facilitate the immobilization process [36]. Thus, lactobacilli along with enterococci may actively compete with *E. coli* for the binding sites located on the surface of the intestinal enterocytes [37], what was noticed in this study (Fig. 6). The interesting tendency that was observed concerned the higher adhesion rate of total bacteria along with the higher number of adhered lactic acid bacteria than *E. coli* triggered by the hydrolysate in comparison to the control culture (Fig. 6). Observed phenomena were a probable result of several mechanisms. Although the growth of *E. coli* was stimulated by analysed substrates (Fig. 3A), it is very likely that when incubated with *L. acidophilus* and *E. faecium*, their proliferation rate is hindered by an unfavourable environmental shift brought about by its acidification evoked by the metabolism of lactic acid bacteria. In consequence, there may be a lowered number of *E. coli* competing for the receptors present on the surface of Caco-2 cells. Additionally, the increasing number of lactobacilli and enterococci might have resulted in the synthesis of substances displaying antibacterial properties thus hindering the *E. coli* adhesion. Moreover, the buckwheat protein hydrolysate facilitated bacterial adhesion with the high number of immobilized beneficial lactobacilli as showed on the Fig. 5C. That may in consequence promote strengthening the intestinal barrier as well as facilitate bacterial enzymatic degradation of nutrients present in the small intestine.

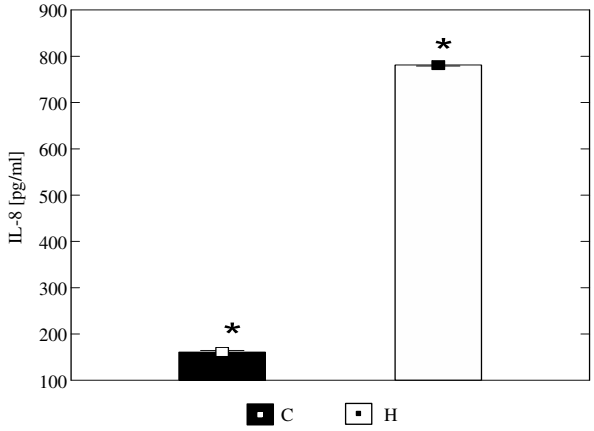

Fig. 8. Interleukin 8 secretion (pg/ml) by Caco-2 cells exposed to the buckwheat protein hydrolysate (H). C – control culture without any protein hydrolysates supplementation. The control and the treated cells demonstrate statistical differences at $P < 0.01$

Buckwheat grains contain also other substances that are able to influence the adhesion of microbiota to intestinal mucosa, such as D-fagomine, non-digestible glucose analog, naturally present in buckwheat grain. Gomez *et al*. [38] demonstrated that it significantly inhibited the adhesion of bacteria from the Enterobacteriaceae family and stimulated the adhesion of *Lactobacillus acidophilus* to intestinal mucosa. The induction of metabolic pathways in bacteria is driven differently by proteins and carbohydrates, similarly as their adhesive potential. Therefore, it is difficult to speculate whether D-fagomine, if remained in the studied protein extract, influenced the biological effect exerted by analyzed hydrolysates. Studies concerning the impact of D-fagomine, D-fagomine-protein complex and proteins are required to scrutinize this topic.

Interestingly, the buckwheat protein hydrolysate hindered the mitosis process of the epithelial cells (Fig. 7) along with the increase in the IL-8 secretion (Fig. 8). Such phenomena may lead to the weakening of the intestinal barrier by impeding its renewal. The significant increase in the secretion of IL-8 from enterocytes may trigger the inflammatory process of the detrimental effect on the gut ecosystem. Secretion of IL-8 by epithelial cells has been suggested to be important in induction of migration of inflammatory cells into the mucosa. The hindrance of enterocytic proliferation combined with IL-8 secretion was previously speculated to be possible stimuli for leaking of the intestinal barrier and translocation of pathogenic and opportunistic bacteria as well as food allergens, thus triggering or deep-

ening the inflammation [39]. Homeostasis of the human gastrointestinal ecosystem encompasses close cooperation of microbiota and intestinal epithelium. Thus, in spite of the enterocytic proliferation hindrance and proinflammatory cytokine secretion by the examined substrates, their positive impact on bacteria may compensate the effect on the epithelial cells in order to maintain the intestinal homeostasis.

Recapitulating, a diet enriched with the buckwheat protein hydrolysates or buckwheat protein hydrolysates themselves liberated after the gastric pepsin hydrolysis may beneficially modulate bacterial flora of the small intestine, stimulating the proliferation and survival rate and adhesion of lactic acid bacteria that in turn may compensate the negative impact on the physiological response of enterocytes. In consequence, such an effect may influence human health in a positive way.

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