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Evaluation of Bean and Soy Tempeh Influence on Intestinal Bacteria and Estimation of Antibacterial Properties of Bean Tempeh

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Abstract

In this study the effect of bean tempeh on the growth of *Bacillus subtilis*, *Escherichia coli*, *Lactobacillus acidophilus* and *Lactobacillus paracasei* bacteria was investigated. Antibacterial activity was observed only in relation to the bacteria *Bacillus subtilis*. The effect of tempeh products on human intestinal microflora was also assessed. Bean and soy tempeh were culinarily processed and next digested in conditions simulating the human digestive tract (one of the digestive tracts was equipped with a mechanism simulating absorption). Soy tempeh stimulated most the growth of bacteria of the genus *Bifidobacterium*, while bean tempeh that of *Escherichia coli*. Using simulation of absorption for the digestion of fried soy tempeh resulted in a higher rise in the bacteria count of the genus *Lactobacillus*, while after digestion of fried bean tempeh the highest increase was recorded for *Bifidobacterium* and *E. coli*.

Key words: tempeh, human intestine microflora, digestion, antibacterial activity

Introduction

Tempeh is a traditional, fermented product made from legume seeds which originates from Indonesia. *Rhizopus oligosporus* fungus strains are used most commonly in the fermentation of tempeh (Nout *et al.*, 2005). Interest in this product has been on the rise in various parts of the world due to its high nutritive value and easy preparation for immediate consumption (convenience food). Tempeh's beneficial effects in counteracting diarrhea in monogastric mammals have been described in the literature. So far, this effect after soy tempeh consumption has been observed in humans (Karyadi and Lukito 2000), rabbits (Karyadi and Lukito 1996; Karmini *et al.*, 1997) and pigs (Kiers *et al.*, 2003). These anti-diarrheal features of tempeh may be related to its antibacterial properties. The first reports on the antibacterial properties of extracts from a substrate fermented by the fungus *R. oligosporus* appeared in 1969. Different microorganisms, *i.e. Streptococcus cremoris* (Wang *et al.*, 1969) or *Bacillus subtilis* (Kobayasi *et al.*, 1992), were indicated as most susceptible to the activity of this type of antibacterial substances. Later studies on the antibacterial activity of soy tempeh extracts showed slight antibacterial activity in relation to *B. subtilis* and *Bacillus stearothermpohilus* (Kiers *et al.*, 2002). Peptides which exhibited antibacterial activity in relation to *Bacillus cereus* were also found in tempeh (Roubos *et al.*, 2008). However, tempeh isolates were not shown to be able to inhibit the growth of *Escherichia coli* which causes diarrhea in humans and animals (Kiers *et al.*, 2002). *In vitro* studies found that tempeh isolates inhibited the cell adhesion of an enterotoxic strain of *E. coli* to intestinal epithelium cells. However, there are no data indicating which of the substances contained in the tempeh affected this process.

The aim of this research was evaluation of antibacterial properties of bean tempeh and comparing impact of bean and soy tempeh on intestine bacteria. No data on the antibacterial properties of bean tempeh have been found. This study analyzed the influence of soy tempeh and bean tempeh on intestinal microflora with the use of an *in vitro* digestion model. In the studies published to date, the effect of tempeh products on intestinal microflora has not been described. We evaluated the thesis that antibacterial properties and anti-diarrhea

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features are connected and if there is a possibility of replacing soy by beans for the production of tempeh without changing their properties against bacteria.

Experimental

Materials and Methods

Microorganisms. The *Rhizopus oligosporus* NRRL 2710 fungus strain came from the Northern Regional Research Laboratory, Peoria, Illinois, USA. *Bacillus subtilis* DSMZ 347, *Lactobacillus paracasei* subsp. *paracasei* DSMZ 20213 and *Lactobacillus acidophilus* DSMZ 20079 bacteria were obtained from Deustche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. *Escherichia coli* 94 (isolated from the human intestine) came from the Institute of Animal Reproduction and Food Analyses, Polish Academy of Sciences, Olsztyn, Poland.

Production of tempeh. The inoculum for soy and bean seed inoculation was a suspension of *R. oligosporus* spores cultured on a PDA medium for 72 h. The seeds of soy (*Glycine max*) cv. Noviko and bean (*Phaseolus vulgaris*) cv. Igołomska, after hulling and boiling (40 min and 20 min, respectively), were inoculated with *R. oligosporus* spores and fermented at a temperature of 37°C for 24 h.

Microbiological media. *B. subtilis* DSMZ 347 bacteria were cultured in DSM broth (meat extract 0.3%, peptone 0.5%, agar 1.5%). *E. coli* 94 was cultured in a medium containing enriched broth at 1.5%, glucose 1.0% and agar 1.5%. *L. paracasei* subsp. *paracasei* DSMZ 20213 and *L. acidophilus* DSMZ 20079 were cultured in MRS medium (yeast extract 0.4%, meat extract 0.8%, peptone K 1.0%, glucose 2.0%, ammonium hydrogen citrate 0.2%, dipotassium phosphate 0.2%, sodium acetate 0.5%, magnesium sulfate heptahydrate 0.02%, manganese sulfate tetrahydrate 0.005 g, Tween 80 0.1%).

The following selective media to determine changes in the counts of intestinal bacteria were applied: the Garsh medium (peptone K 2.0%, agar 1.5%, lactose 1.0%, sodium acetate 0.6%, yeast extract 0.5%, lithium chloride 0.3%, $Na_2HPO_4 \times 12\ H_2O$ 0.25%, K_2HPO_4 0.2%, L cysteine 0.04%, $MgSO_4 \times 7\ H_2O$ 0.012%) for *Bifidobacterium*; agar with kanamycin, aesculin and sodium azide (Merck) for bacteria from the genus *Enterococcus*; McConkey's medium (peptone K 1.7%, meat peptone 0.3%, bile salts 0.15%, NaCl 0.5%, lactose 1.0%, methyl violet 0.0001%, neutral red 0.003%, agar 1.3%) for *E. coli*; and the MRS medium for bacteria from the genus *Lactobacillus*.

Bacterial culture conditions. The bacteria *B. subtilis* were cultured on agar slants at a temperature of 30°C for 48 h. Next they were washed off the slants with10 ml of physiological saline, followed by washing into 90 ml culture broth with no agar addition.

Further culture was run on a shaker in Erlenmeyer flasks at 120 rpm at 30°C for 24 h. *E. coli* and *Lactobacillus* were cultured on agar slants at 37°C for 48 h. Next the bacteria were washed off the slants with 10 ml of physiological saline, followed by washing into 90 ml of the appropriate medium with no agar added. Further culture was run in Erlenmeyer flasks at 37°C for 24 h. The number of cells was 8.7, 8.2, 7.7, and 7.3 log c.f.u./ml for *B. subtilis*, *E. coli*, *L. paracasei*, and *L. acidophilus*, respectively.

Preparation of intestinal microflora. Fecal specimens were collected from three healthy individuals (2 women and 1 man), aged 20–28 years old, who had not been treated with antibiotics for at least 3 months prior to the specimen collection and had not reported digestive problems. The fecal specimens were placed under anaerobic conditions immediately after excretion. An identical amount (w/w) of phosphate-carbonate buffer at a temperature of 37°C was added to each individual's fecal specimen (Barry *et al.*, 1995). Next the entire mass was homogenized, combined at equal volumes and sieved through a sieve with mesh size of 1 mm. These procedures were performed in a nitrogen atmosphere (Aura *et al.*, 2005).

Preparation of extracts from tempeh. An amount of 10 g of tempeh was homogenized (homogenizer H-500; Pol-EkoAparatura) with 20 ml of water for 3 minutes at 1500 rpm. The homogenisate was filtered through a sterile filter with pore size of 0.22 μm (Millipore). The pH of extracts was measured.

Determining antibacterial activity by agar well and paper discs methods. A 1 ml culture of bacterial cells was flooded with a medium specific for each bacterial strain with an addition of agar. The volume of 0.1 ml tempeh extract was poured into a well 0.5 cm in diameter. For the paper disc analysis, discs saturated with tempeh extract were placed on medium surfaces. Plates inoculated with *B. subtilis* were incubated at 30°C, while the other tested bacteria were cultured at 37°C. After 24 h, the bacterial growth inhibition zone around the well or disc was measured.

Determining antibacterial activity by turbidimetry. The culture was run in test tubes, where 1 ml of bacterial suspension and 0.5 ml of tempeh extract were added to 8.5 ml of the respective medium. Evaluations were taken at 2/3 of the log growth phase of a given indicator strain. Absorbance was measured at a wavelength of 650 nm on a spectrophotometer (Helios Delta, Thermo Scientific, USA). Dependence curves of absorbance were determined based on the amount of tested bacterial cells.

Digestion under *in vitro* conditions. The *in vitro* digestion model consisted of three simulation stages:

the stomach, small intestine and large intestine. Tempeh after culinary processing (10 min boiling or 5 min frying in rapeseed oil at 170–180°C) was comminuted, weighed into 6 g samples, mixed with 60 ml water and placed in 100 ml flat-bottomed flasks.

Using hydrochloric acid, the mixture was brought to pH 2, next a solution of pepsin was added. After 4 h sodium hydrogencarbonate was added, then intestinal-pancreatic extract was added when the pH reached 6. Next, using sodium hydrogencarbonate, the pH was raised to 7.4 and intestinal microflora were added. After 2.5 h the pH was set at 8.0 and maintained for 18 h. When pH was established at 8.0, vessels with the contents being digested were blown with nitrogen (Gumienna *et al.*, 2007).

***In vitro* digestion model with simulation of active transport.** The digestion process using *in vitro* simulation of active transport was run identically to conventional digestion up to the stage simulating digestion in the small intestine. Fried tempeh was subjected to digestion under such conditions for 24 h. After introducing intestinal-pancreatic extract as well as intestinal microflora and stabilizing pH, the digested contents were transferred to a set containing a membrane with 3.5 kDa pore size (DispoDialyzer, SpectrumLabs). The closed set was placed in a water bath at pH of 7.4 and temperature of 37°C. After 2.5 h the pH was raised to 8.0 using sodium hydrogencarbonate. The process was stopped after 18 h.

Evaluation of changes in intestinal microflora. Changes in microorganism counts of selected intestinal microflora groups after the stages of digestion were evaluated by plate culture on selective media according to the Koch method (Goderska *et al.*, 2008). The results were expressed as cell count increase, *i.e.* the difference between the number of bacterial cells after digestion and the number of bacterial cells at the beginning of digestion (increments).

Statistical analysis. Statistical analysis was carried out using Microsoft Excel 2003 and Statistica 8.0 StatSoft software. All experiments were performed with at least three replicates. The least significant difference (LSD) test and Tukey's test were used to verify differences between the samples.

Results and Discussion

Antibacterial properties. To date, the antibacterial properties of soy tempeh have been mainly described in the literature (Kiers *et al.*, 2002; Roubos *et al.*, 2008). This study focused on determining such properties in bean tempeh. The tested strains used to determine the antibacterial properties of tempeh were bacteria which can colonize the human intestine (*E. coli*, *L. acidophilus* and *L. paracasei* subsp. *paracasei*) as well as *B. subtilis*, which does not colonize the human digestive tract but its particular sensitivity to the antibacterial agent from *R. oligosporus* post-culture media was reported (Kobayasi *et al.*, 1992; Kiers *et al.*, 2002). When applying the bean tempeh extracts to determine antibacterial activity using the paper disc and well methods, no such activity was observed in relation to the tested bacterial strains. Kiers *et al.* (2002), using the disc method, recorded antibacterial activity of soy tempeh against *Bacillus stearothermophilus* and *B. subtilis*. The tempeh extracts obtained by these researchers did not exhibit antibacterial activity in relation to *E. coli*, and only one of the *R. oligosporus* strains used in tempeh fermentation showed activity against *B. subtilis*. However, the tempeh extracts caused an inhibition of *B. stearothermophilus* growth.

The lack of antibacterial activity in the bean tempeh extracts, determined using both the well and paper disc methods, could have been caused by a lower amount of substances displaying antibacterial activity against the tested bacteria forming in the bean tempeh in comparison to the soy tempeh (Kiers *et al.*, 2002).

In this study, using turbidimetry, the antibacterial activity of bean tempeh extracts was determined only in relation to *B. subtilis* (Fig. 1). Kobayasi *et al.* (1992) obtained similar results: by using the turbidimetric method they showed the antibacterial activity of the *R. oligosporus* IFO 8631 post-culture medium on a liquid solution of casein hydrolysate against the *B. subtilis* strains, whereas they did not find such activity against *E. coli*.

The aim of applying tempeh extracts, determined by the turbidimetry method, differing in fermentation time (samples taken every 4 h) was to identify the moment of antibacterial substance formation in the tempeh. The presence of an antibacterial agent against the bacteria *B. subtilis* could be detected in bean tempeh only after the 12th hour of fermentation (Fig. 1), which was the time of visible hyphae of the fungus *R. oligosporus* forming on the seeds. No effect of the tempeh extracts on the growth of *Lactobacillus* and *E. coli* was observed. Tempeh extracts did not contain organic acids which could inhibit growth of *B. subtilis* cells number. The pH value did not change significantly during the 24 h of tempeh fermentation. No publication describing the effect of tempeh on bacteria from the genus *Lactobacillus* was found in the literature.

So far, two substances originating from the *R. oligosporus* post-culture media and displaying antibacterial activity have been described. One of these was a protein of 5.5 kDa, isolated from the *R. oligosporus* culture on a substrate of casein hydrolysate (Kobayasi *et al.*, 1992), while the other was a peptide weighing less than 3 kDa found in soy tempeh (Roubos *et al.*, 2008). To date it has not been explained if these proteins were synthesized

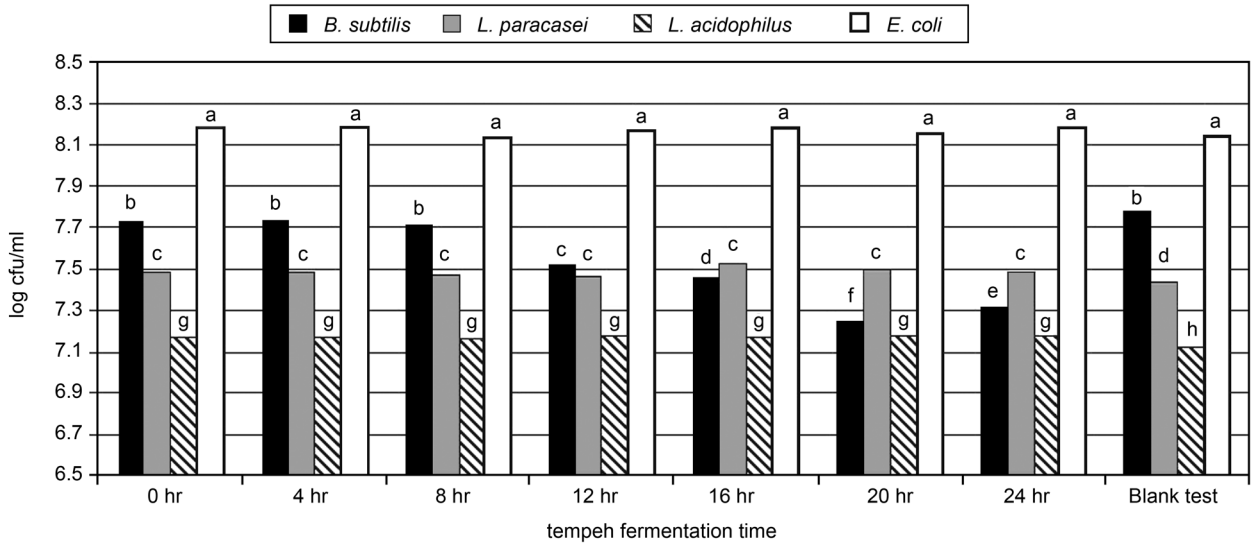

Fig. 1. Antibacterial properties of extracts from bean tempeh fermented for 4 to 24 hours expressed as the decrease of the number (log cfu/ml) of test bacteria. Bars marked with different letters differ significantly ($P < 0.05$; LSD test).

by *R. oligosporus* or whether they could have formed as the result of activity of the enzymatic apparatus of filamentous fungi through the hydrolysis of proteins contained in the fermented substrate.

In the analyzed literature, apart from the antibacterial properties of soy tempeh, antibacterial activity was described only for pea tempeh. Nowak and Steinkraus (1988) evaluated the effect of pea tempeh on the growth and metabolism of *Clostridium* by using turbidimetry and measuring the amount of gas produced by the tested bacteria. No other publications describing the antibacterial properties of tempeh produced from raw materials other than soy have been found.

Intestinal microflora. Despite reports of tempeh's positive effect in counteracting the onset of diarrhea, to date the influence of tempeh products on intestinal microflora has not been described. We decided to verify whether these properties could be in any way connected with tempeh's influence on the microflora of the human digestive tract.

After 24 h of fermentation, bean and soy tempeh were subjected to digestion in conditions simulating the human digestive tract. Human fecal microflora were added at the stage corresponding to the small intestine and an increase in the number of microorganisms belonging to the genera *Bifidobacterium*, *Lactobacillus*, *E. coli* and *Enterococcus* was determined at the end of the digestion process.

Some authors consider McConkey's selective medium, applied as one to proliferate *E. coli* bacteria, as a medium for the proliferation of bacteria from the group of *Enterobacteriaceae* (Goderska *et al.*, 2008). However, the manufacturer of this medium (BTL) recommends it as suitable for the proliferation of *E. coli*, *Salmonella* and *Shigella*, which belong to the *Enterobacteriaceae*. Due to the considerable dominance of *E. coli* bacteria over the other microorganisms colonizing the human digestive tract (Marteau et al. 2001) capable of growing on McConkey's medium, bacteria growing on this medium were described as those belonging to *E. coli* bacteria.

The results of determining the effect of tempeh-type products on intestinal microflora are presented as an increment in the count of cells after the completion of digestion in relation to the number of bacterial cells added at the beginning of digestion simulating the small intestine. This was caused by the necessity to ensure a lack of dependence on the different numbers of bacterial cells in the suspension formed from the material obtained at different time from the fecal microflora donors.

The digestion process of soy tempeh intensified the growth of *Bifidobacterium* and *E. coli* cells number, while bean tempeh rather stimulated the growth of *E. coli* and *Enterococcus* (Fig. 2). A higher increase in the counts of the tested bacteria was visible after digestion of cooked tempeh products than fried tempeh. Cooked beans and soy most stimulated the growth of number of bacterial cells from the genus *Enteroccocus* and *E. coli* and differences between stimulation of this group and *Lactobacillus* and *Bifidobacterium* was the largest in comparison with theses differences observed after digestion of other analyzed products. In digestion of the blank sample, where no product was added apart from water, a decrease was observed in the number of cells of the tested types of intestinal microflora, except for *Enterococcus*.

Applying the model simulating the transport of low molecular components to digest fried tempeh increased the stimulation of microflora growth (Fig. 3). Soy tem-

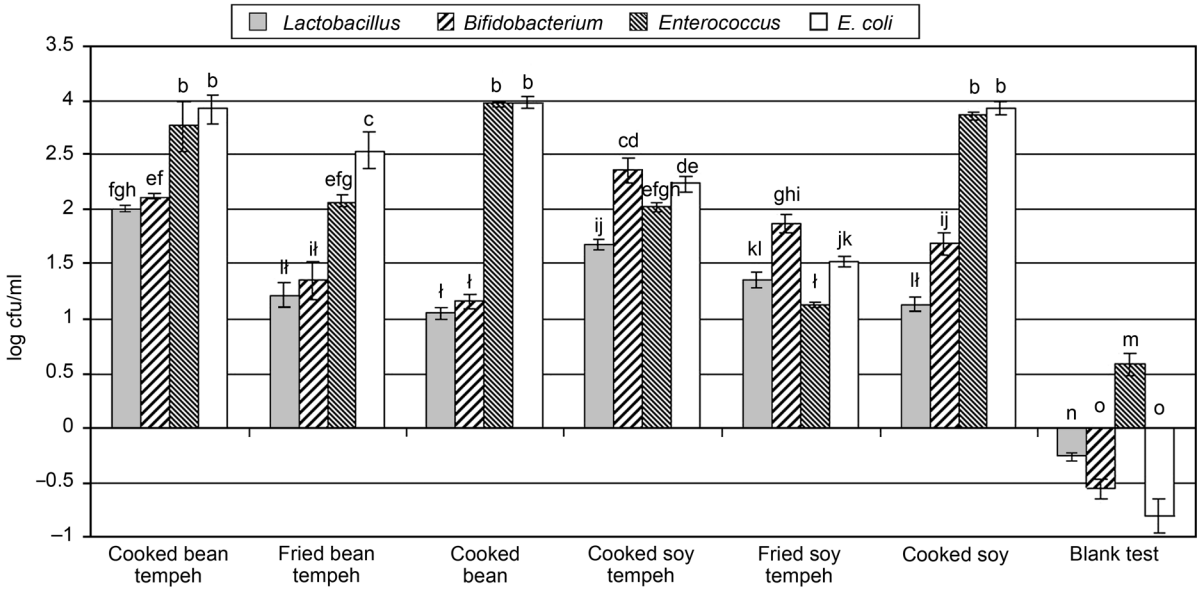

Fig. 2. Increments in the number of cells of intestinal microflora after digestion of tempeh products. Bars marked with different letters on the Fig. 2 and Fig. 3 differ significantly ($P < 0.05$; Tukey's test).

peh stimulated most the growth of bacteria from the genus *Lactobacillus*, and bean tempeh that of bacteria from the genera *Bifidobacterium* and *E. coli*. With the exception of the *Lactobacillus* bacteria, no statistically significant differences were found in growth stimulation of other marked types of intestinal microflora during digestion of both fried bean and soy tempeh. Applying the model simulating the transport of low molecular components to digest fried tempeh products, in comparison to the typical digestive system, led to increased growth of health-promoting intestinal microflora.

There have been many reports that after the consumption of soy tempeh the duration and incidence of diarrhea in humans (Karyadi and Lukito 2000) and animals (Karyadi and Lukito 1996; Karmini *et al.*, 1997; Kiers *et al.*, 2003) subsided. However, the mechanism behind this has not been clarified to date. It seems that it is not related to tempeh's antibacterial activity since in most cases thermal processing deactivated the antibacterial agent. Nowak and Steinkraus (1988) stated that subjecting pea tempeh to 10-minute cooking resulted in the complete disappearance of antibacterial properties against *Clostridium perfringens*. Roubos *et al.* (2008) observed the lack of antibacterial properties of a peptide isolated from tempeh after heating to 60°C. Only Kobayasi *et al.* (1992) observed the antibacterial agent's resistance to temperature, but they tested a protein isolated from casein hydrolysate used in the *R. oligosporus* culture. The antibacterial agent isolated by Roubos *et al.* (2008) was susceptible to the action of proteases, thus digestive enzymes can also degrade the antibacterial peptides present in soy tempeh.

Other authors observed the effect of soy tempeh extracts on a decrease in adhesion of an enterotoxic

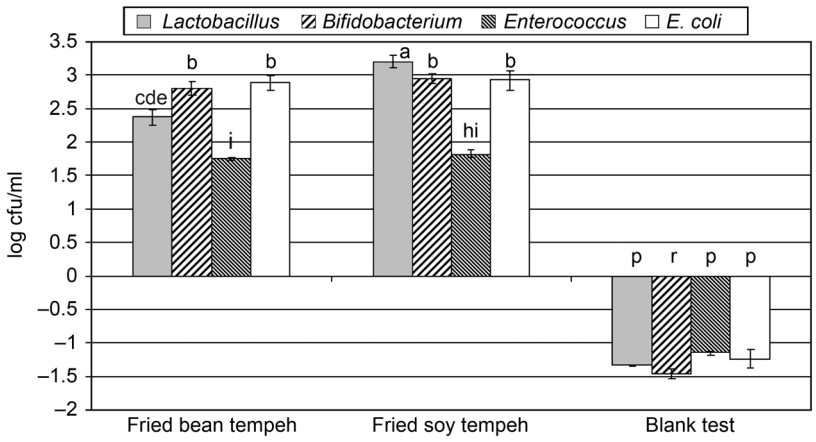

Fig. 3. Increments in the number of cells of intestinal microflora after digestion of tempeh products in the system with simulation of absorption of low melecular subtances.
Bars marked with different letters on the Fig. 2 and Fig. 3 differ significantly ($P < 0.05$; Tukey's test).

strain of *E. coli* to the intestinal epithelium cells of pigs (Kiers *et al.*, 2002) and humans (Roubos *et al.*, 2009), suggesting that this could be the antidiarrheal mechanism activated after tempeh consumption. However, tempeh used in the above-mentioned study was subjected neither to culinary processing nor to the digestion process, and these processes may to reduce these properties.

So far, an increase in intestinal bacteria counts has not been described during digestion of tempeh products. Stimulating growth of bacteria colonizing digestive tract microflora may comprise one of the mechanisms restoring the balance in the intestinal biotope and thus relieving the symptoms of diarrhea. Applying the *in vitro* digestion model stimulating absorption, which is more similar to conditions in the intestine, caused a growth of positive for human health microflora groups, however further studies on animals are necessary to confirm these results.

In conclusion, bean tempeh displayed antibacterial properties against *B. subtilis*, and no growth inhibition was observed in the remaining tested bacteria: *E. coli*, *L. acidophillus* and *L. paracasei*. When assessing intestinal bacteria counts in the *in vitro* digestion model, it was shown that tempeh products stimulated the growth of these bacteria, especially those from the genera *Bifidobacterium* and *E. coli*. Applying the model simulating the transport of low molecular components to *in vitro* digestion played a significant role in the increase of intestinal microflora counts. One of the mechanisms of tempeh products that prevents diarrhea might be the observed stimulation of growth of certain intestinal microflora groups.

Acknowledgements

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