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Viability and growth promotion of starter and probiotic bacteria in yogurt supplemented with whey protein hydrolysate during refrigerated storage*

Przeżywalność oraz stymulacja wzrostu kultur jogurtowych i probiotycznych w jogurtach wzbogaconych hydrolizatem białek serwatkowych podczas ich chłodniczego przechowywania

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Summary

The effect of whey protein hydrolysate (WPH) addition on growth of standard yoghurt cultures and *Bifidobacterium adolescentis* during co-fermentation and its viability during storage at 4°C in yoghurts has been evaluated. WPH was obtained with the use of serine protease from *Y. lipolytica* yeast. Stirred probiotic yoghurts were prepared by using whole milk standardized to 16% of dry matter with the addition of either whey protein concentrate, skim milk powder (SMP), WPH-SMP (ratio 1:1), WPH. The hydrolysate increased the yoghurt culture counts at the initial stage of fermentation and significantly inhibited the decrease in population viability throughout the storage at 4°C in comparison to the control. The post-fermentation acidification was also retarded by the addition of WPH. The hydrolysate did not increase the *Bifidobacterium adolescentis* counts at the initial stage. However, the WPH significantly improved its viability. After 21 days of storage, in the yogurts supplemented with WPH, the population of these bacteria oscillated around 3.04 log₁₀ CFU/g, while in samples where SMP or whey protein concentrate was used, the bacteria were no longer detected.

Keywords: *Bifidobacterium* • whey protein hydrolysate • *Yarrowia lipolytica* • cell viability

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INTRODUCTION

Yogurts and other fermented milk beverages have been used as the most popular products for the incorporation of probiotic microorganisms that are believed to provide health benefits when consumed [28]. Studies have demonstrated the benefit of probiotic bacteria in the treatment of intestinal disorders, rotavirus diarrhoea, food allergies and colonic disorders [19,23]. In vitro studies conducted by Gilliland [7] showed that Bifidobacteria and *L. acidophilus* are able to utilize cholesterol in growth media. In order to exert a positive influence on the host, the viability of the strain is of utmost importance. The strains used for therapeutic benefits should originate from human sources, be capable of surviving in acidic fermented products, and be available in sufficient numbers in the product prior to consumption. The recommended minimum count is 10^6 live organisms [8]. Most of the probiotic yogurts include live strains of *L. acidophilus* and species of Bifidobacterium in addition to the conventional yogurt organisms [8]. Bifidobacteria, a predominant genus in the stool of breast-fed infants, is widely used as a probiotic dietary additive with beneficial health properties. Bifidobacteria are only used in fermented dairy products to a limited extent because of their slow growth in that medium and a loss of viability during storage. However, milk contains all the essential nutrients to support the extended growth of bifidobacteria, apart from free amino acids and small peptides which are present in insufficient concentrations [9]. Strain survival depends on pH (and buffering capacity), dissolved oxygen, the presence of competing microorganisms, storage temperature and the presence of microbial inhibitors in the food matrix. The optimum pH for growth is 6-7, with no growth below 4.5 or above 8.5. The optimum temperature is 37°C, with maximum growth at 43°C and no growth below 25°C [18]. Some practices have been proven to be able to increase the survival of these bacteria in yogurt products. Substances such as oligosaccharides and non-protein nitrogen can improve Bifidobacteria growth [29]. Vitamins, dextrin and maltose were also found to stimulate the growth of Bifidobacteria species in milk, while sucrose and iron salts had little effect [8]. Milk supplementation with peptides and amino acids may also increase the viability of probiotic organisms. Supplementation with casein and whey protein hydrolysates decreased the complex viscosity and fermentation time in yoghurts. The growth-promoting activity of bovine kappa-casein on *B. longum* was ascribed to its conjugated cystine residues [21]. The major whey proteins, alpha-lactalbumin and beta-lactoglobulin, were also found to be excellent growth promoters [10]. It therefore appears important to deliver adequate peptides and amino acids for the improved growth and viability of these bacteria. Apart

from that, it is necessary to develop hydrolysates with better quality at a lower cost.

Whey proteins are generally resistant to hydrolysis, and the use of enzymes significantly increases the cost of their production [15]. One promising alternative is the use of an extracellular serine protease, isolated from *Yarrowia lipolytica* yeast originating from mold cheese, for the hydrolysis of whey proteins, resulting in a deep degradation [2]. The aim of this study was then to investigate the effects of the whey protein hydrolysate obtained using serine protease from *Y. lipolytica* yeast, on the growth of *Bifidobacterium adolescentis* during co-fermentation with regular yoghurt cultures and the corresponding viability during cold storage.

MATERIALS AND METHODS

Substrates: Whey protein concentrate (WPC-80), manufactured from sweet whey and spray-dried, was provided by Davisco Foods International, Inc. Skim milk powder (SMP) was provided by Mlekovita.

Yoghurt culture and probiotic strain: YoFlex® starting culture was supplied by Chr. Hansen. *Bifidobacterium adolescentis* strain was purchased from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany, <http://www.dsmz.de>). Bifidobacterium strains were routinely grown for 48 h in anaerobic conditions at 37°C in MRS broth, modified by the addition of cysteine hydrochloride at 0.05%, in anaerobic jars (Merck). Each culture was centrifuged at 8000 G for 10 min at 4°C. The pellets were washed twice, resuspended in Ringer solution and used as inoculum.

Hydrolysate preparation: Yeast serine protease was obtained from *Yarrowia lipolytica* J111c from the culture collection of the Department of Biotechnology and Food Microbiology, Wrocław University of Environmental and Life Sciences, where it had been previously isolated from a "Rokpol" cheese. Proteolytic activity of the serine protease was determined with the use of 2% casein as a substrate in 0.1M Tris-HCl at pH 8.0 [3]. Enzymatic hydrolysis of a 10% solution of WPC-80 was conducted with the enzyme introduced at 150 U/g of hydrolyzed protein. The reaction was carried out at 37°C for 24 hours in 0.1 M Tris-HCl buffer at pH 8.0. Hydrolysis was terminated by thermal inactivation (for biological activity determinations) or by the addition of 10% trichloroacetic acid (TCA) (1:1 V/V). WPC-hydrolysate (WPH) was further vacuum concentrated.

Determination of protein content: Protein content was determined by colorimetric method [16], using BSA (Sigma, P0834) as a standard.

Degree of hydrolysis: The course of the hydrolysis was monitored by the determination of soluble peptide concentration in 5% TCA in relation to total protein. The concentration of the trichloroacetic acid-soluble product in the supernatant was measured spectro-photometrically at $\lambda=280$ nm [24].

Free amino group content: The content of free amino groups ($\mu\text{mol Gly/g}$) was determined using trinitrobenzene sulfonic acid (TNBS, Sigma) according to Kuchro et al. [11]. Samples of hydrolysate were diluted with 0.1 mol/L borate buffer to a final volume of 2 mL, then mixed with 50 μl of TNBS reagent (0.03 mol/L) and incubated in the dark for 2 h at room temperature. The reaction was stopped by adding 2 mL of 0.1 mol/L sodium phosphate containing 1.5 mmol/L sodium sulfate, and the absorbance was measured spectro-photometrically at $\lambda=420$ nm. The results were expressed as $\mu\text{mol Gly/g}$ in relation to a standard curve prepared with the defined concentration of glycine.

Reversed-phase high performance liquid chromatography (RP-HPLC): Peptide profiles were determined by RP-HPLC with an Agilent 1100 Series system. The peptide preparations were solubilized in even volumes of phase A (0.1% TFA in H_2O) before loading onto the chromatographic HPLC column (Zorbax Eclipse XDB-C18 Agilent column (50 x 4.6 mm). Separation was performed at a flow rate of 1 ml/min at 30°C. Peptide fractions varying in hydrophobicity, were eluted from the column in a linear gradient of phase B (0.1% TFA in acetonitrile) and collected and lyophilized. Absorbance measurement was made at $\lambda=230\text{nm}$ (DAD, G1315B).

Preparation of yoghurts: All cultures were prepared according to manufacturer instructions. Yoghurt starter cultures (Chr. Hansen, Germany) were poured into 1 liter of sterilized milk at 40°C and mixed thoroughly. The mixtures were then put into glass containers and incubated at 42°C until reaching pH = 4.5. After fermentation, the yoghurt samples were cooled and transferred to a refrigerator and stored at 4°C until analysis.

Mixed yoghurt cultures propagated in sterile skim milk were added at 2% (v/v) to pasteurized and cooled milk (43 °C). Cultures of *Bifidobacterium* were grown after incubation at 37°C in anaerobic condition and then centrifuged at 2500 x g, 4°C, 10 min. The pellet was washed twice in a saline solution. The growth evaluation was made by turbidimetric measurement of the OD650 using a spectrophotometer. The suspension was added to the pasteurized and cooled milk (43°C).

Stirred probiotic yoghurt cultures in whole milk were standardized to 16% of dry matter with the addition of WPC-80, SMP, WPH-SMP (ratio 1:1), WPH. After they were mixed properly, each milk base was heated to 85°C for 30 min by circulation in a hot water bath, and then cooled to 43°C in an ice bath. The mixture was incubated under vacuum conditions at 41°C for 5h (Buchi Syncore Q-101).

The resulting yoghurt milk samples were packed in Microlab Crimp vials in an atmosphere of nitrogen.

Viability assay. The samples of probiotic yoghurt milk were stored at 4°C for 21 days. The yoghurt samples were then serially diluted in sterile Ringer solution and pour-plated in duplicate on selected media. Populations of standard yoghurt cultures were enumerated on MRS agar plates and incubated at 37°C for 72 h. Populations of *Bifidobacterium adolescentis* were enumerated on TOS-Propionate agar with a mupirocin (MUP) selective supplement. TOS-Propionate agar plates were incubated anaerobically (anaerobic jars with Anaerocult A gas packs; Oxoid) at 37°C for 72 h.

RESULTS

The course of the enzymatic hydrolysis of WPC-80 was monitored by determination of the degree of hydrolysis [DH]. The DH determined after 24 h hydrolysis reached 31%. During the protein degradation in all hydrolysates, a proportional increase in free amino group content (FAG) was observed. The final concentration of released FAG reached 2970.9 $\mu\text{mol Gly/g}$. The progress of hydrolysis was also confirmed by RP-HPLC peptide profile analysis (Fig. 1). On the chromatograms of the hydrolysates the presence of subfractions were noted, which were then eluted from the column at low concentration of acetonitrile, and were varied in terms of hydrophobicity.

The growth-promoting activities of SMP, WPC-80 and WPH were tested in standard yoghurt cultures and yoghurt cultures co-incubated with *Bifidobacterium adolescentis* (Fig. 2,3). The hydrolysate increased the standard yoghurt culture counts at the initial stage, compared to the control. When the dry matter was established with the addition of SMP and WPC-80, the populations observed were 5.76 and 5.95 \log_{10} CFU/g respectively. On the other hand, the addition of the hydrolysate resulted in a population of 8.72 \log_{10} CFU/g (Fig. 2). In the yoghurts with the mix of WPH and SMP, the highest initial population of 8.95 \log_{10} CFU/g was noticed (Fig. 2).

The counts of the standard yoghurt culture bacteria decreased throughout storage. The decline could be significantly delayed by the addition of hydrolysate. After 21 days of storage at 4°C, in yogurts supplemented with the mix of WPH/SMP the population of 7.86 \log_{10} CFU/g was observed compared to 5.08 \log_{10} CFU/g for SMP.

The pH of the yogurts declined after time. However, the hydrolysates significantly inhibited post-fermentation acidification (Fig. 4). The samples where WPH was used displayed a slower decrease in pH compared to the control samples supplemented with intact proteins.

In yoghurts with starter cultures co-incubated with *Bifidobacterium adolescentis*, hydrolysates still increased the yoghurt culture counts at the initial stage but it was less effective compared to the traditional method which did not include the probiotic strain (Figs. 4,5). The addition

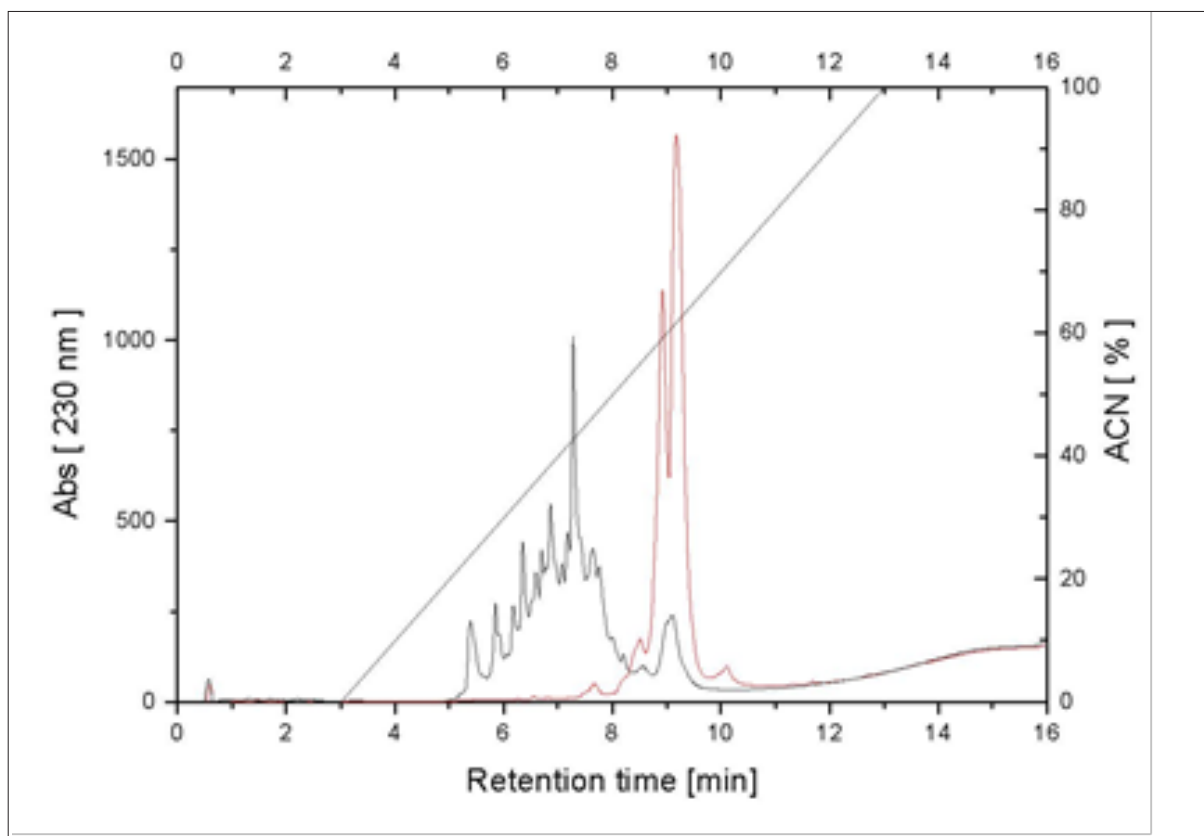


Fig. 1. RP-HPLC profiles of peptide fractions (black) obtained after 24 hours hydrolysis of WPC-80 with serine protease isolated from *Yarrowia lipolytica* introduced at 150 U/g. Undigested 1% protein solution was use as a control of hydrolysis (red)

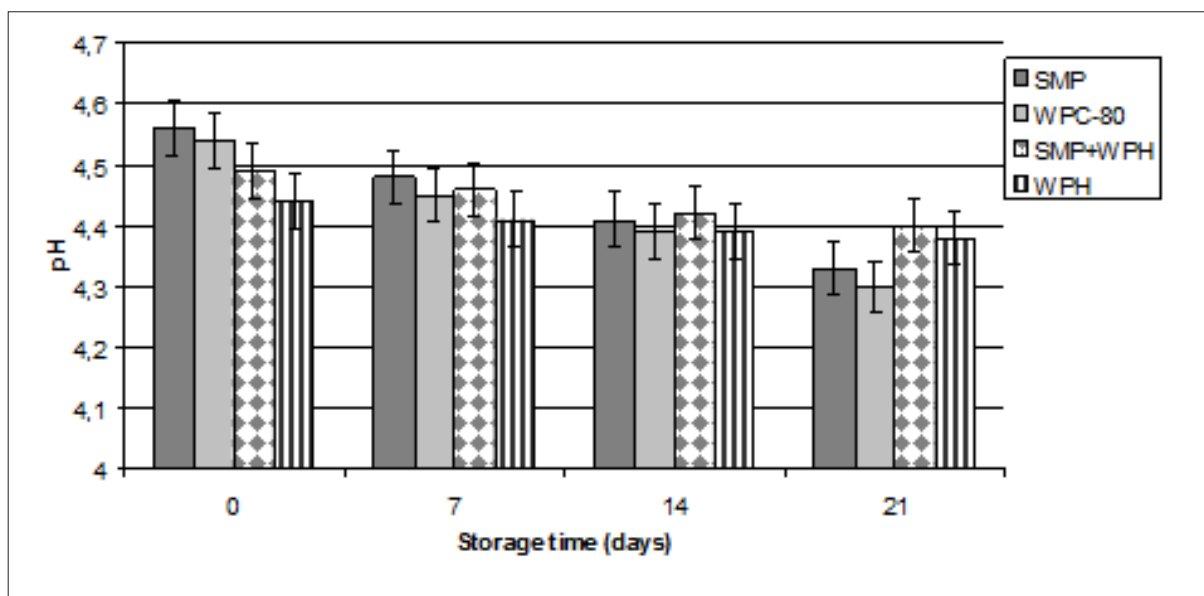


Fig. 2. Changes in pH during storage at 4°C in yoghurts obtained with yoghurt cultures supplemented with skim milk powder SMP, whey protein concentrate WPC-80, skim milk powder + whey protein hydrolysate SMP+WPH, whey protein hydrolysate WPH

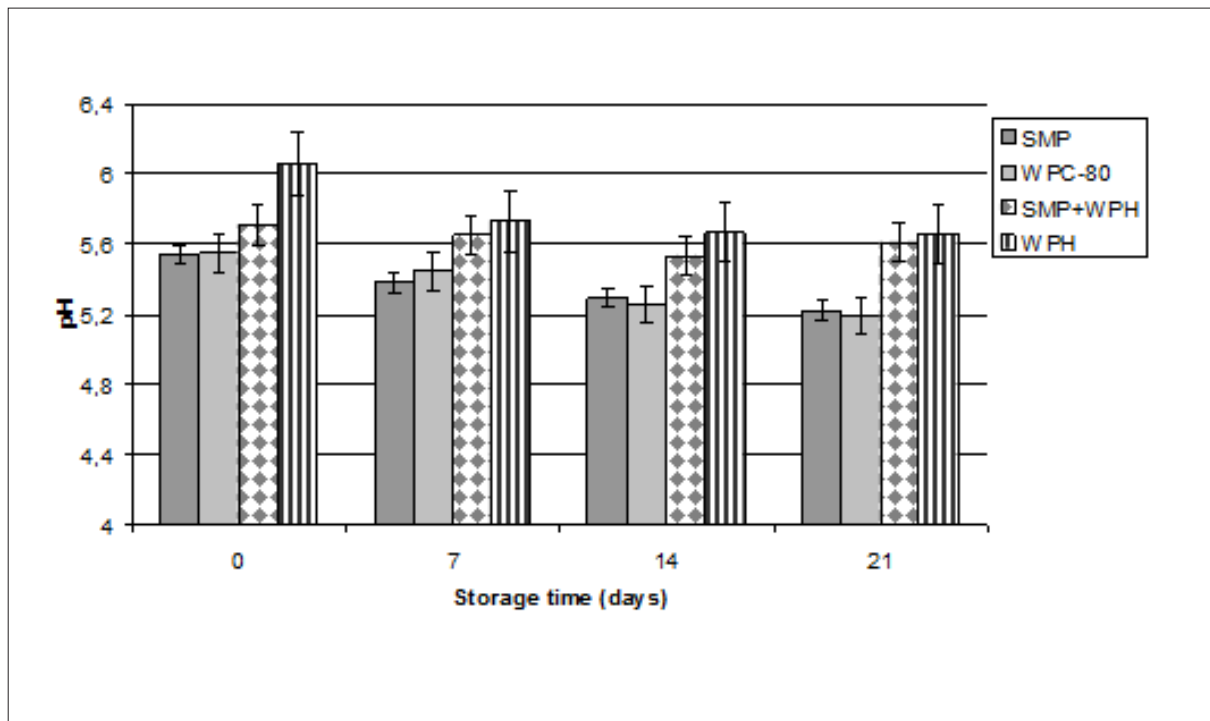


Fig. 3. Changes in pH during storage at 4°C in yoghurts obtained with yoghurt cultures co-incubated with *Bifidobacterium adolescentis* supplemented with skim milk powder SMP, whey protein concentrate WPC-80, skim milk powder + whey protein hydrolysate SMP+WPH, whey protein hydrolysate WPH

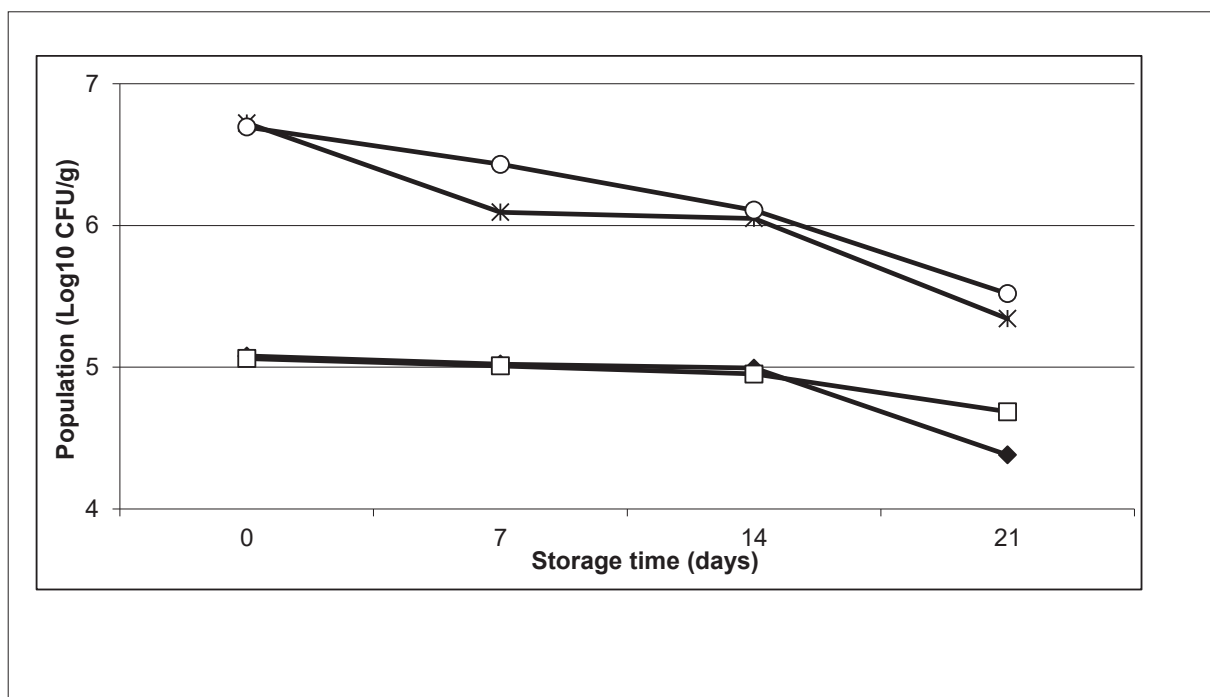


Fig. 4. Viability of yoghurt cultures over a storage period of 21 days at 4°C in yoghurt samples supplemented with: -♦- skim milk powder SMP; -□- whey protein concentrate WPC-80; -*- skim milk powder + whey protein hydrolysate SMP+WPH; -○- whey protein hydrolysate WPH

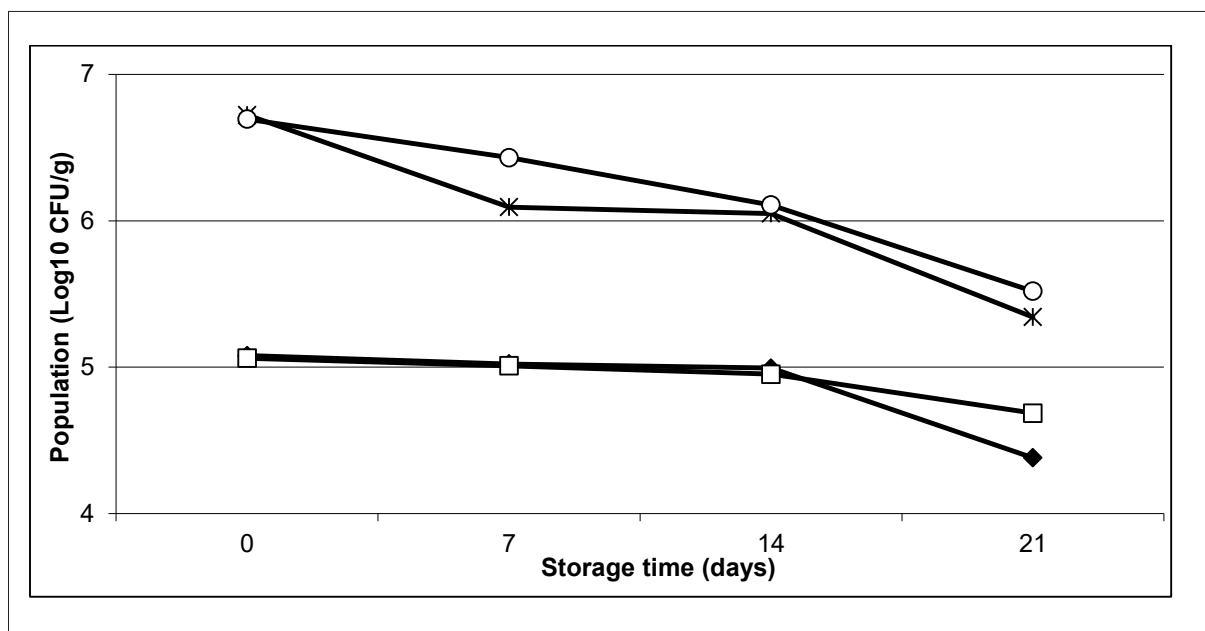


Fig. 5. Viability of yoghurt cultures co-incubated with *Bifidobacterium adolescentis* over a storage period of 21 days at 4°C in yoghurt samples supplemented with: ◆- skim milk powder SMP; □- whey protein concentrate WPC-80; * - skim milk powder + whey protein hydrolysate SMP+WPH; ○-whey protein hydrolysate WPH

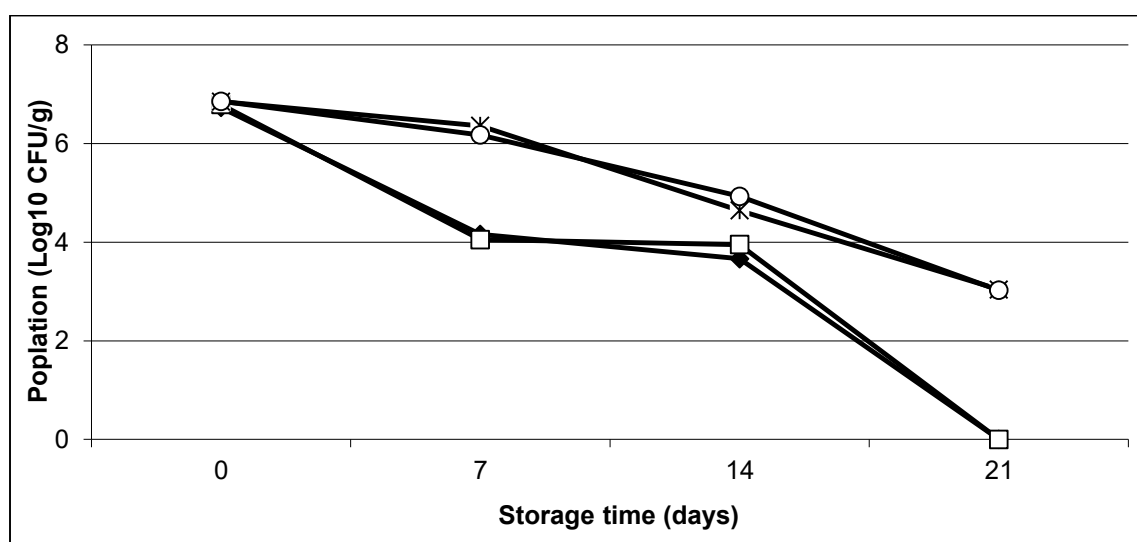


Fig. 6. Viability of *Bifidobacterium adolescentis* co-incubated with yoghurt cultures over a storage period of 21 days at 4°C in yoghurt samples supplemented with: ◆- skim milk powder SMP; □-whey protein concentrate WPC-80; * - skim milk powder + whey protein hydrolysate SMP+WPH; ○-whey protein hydrolysate WPH

of the hydrolysate resulted in the population ranging from 6.69 to 6.76 log₁₀ CFU/g, while supplementing with SMP and WPC-80 the bacteria count was marked at 5.08 and 5.06 log₁₀ CFU/g. This may be affected by the fact that co-fermentation with the probiotic strain resulted in higher pH levels 5.54-6.06 (Fig. 5).

The decline in viable counts of yoghurt culture during the 21 days of storage was less significant in samples

where the hydrolysate was used. The final population ranging from 5.34 to 5.52 log₁₀ CFU/g exceeded the initial population observed in the samples where intact proteins were used as a supplement.

The growth of *Bifidobacterium adolescentis* during co-fermentation with yoghurt cultures is presented in Fig. 6. The hydrolysate did not increase the probiotic culture counts at the initial stage. Regardless of the substrate

used in all samples, the population observed in the beginning was 6.73-6.86 log₁₀ CFU/g.

It is well known that the viability of probiotic bacteria in products during a long refrigerated shelf life is generally unsatisfactory. The counts of probiotic bacteria in yogurts decreased throughout storage (Fig. 6). However, WPH significantly improved the viability of *Bifidobacterium adolescentis*. After 21 days of storage, in yogurts supplemented with the hydrolysate, the population was around 3.04 log₁₀ CFU/g, while in samples with SMP and WPC-80 the bacteria were no longer detected.

DISCUSSION

The hydrolysis of proteins is a widely used technique in the modification of physico-chemical, functional and sensoric properties [4]. Milk protein hydrolysates are intended to be used as additives in food products for improving functional properties, such as foaming and emulsifying capacity, gel formation, viscosity, texture and water-binding capacity, as well as boosting the nutritional characteristics of the final product.

Our study has shown that WPH obtained with the use of serine protease from *Y. lipolytica* yeast exhibits a growth-promoting activity for yoghurt culture bacteria. Enzymatic hydrolysis results in generating peptides and amino acids. Lactic acid bacteria (LAB) require a rich growth media containing compounds such as amino acids, peptides, fatty acids and vitamins. Small peptides can enhance the growth and acidifying activity of probiotics. The effect of a different DH on bacteria growth depends on their proteolytic enzymes and peptide transport system [1]. LAB have different pathways for the transport of free amino acids, di- and tri-peptides and oligopeptides of up to six amino acid residues [24]. The highest rate of uptake is provided by the di- and tri-peptide transporting system [30].

Hydrolysates with a high content of short peptides could be expected to exhibit a significant effect on the growth of LAB [13]. It has been shown that casein degraded with papain increased the growth of probiotic bacteria and increased acidifying activity [29]. Yogurts with the highest DH (26.7%) decreased the fermentation time more significantly. Short coagulation times can improve the growth and survival of probiotic bacteria [29]. Moreover, the observation from our research may be supported by the studies of Kurmann [12], who suggested that an increased amount of thiol groups from whey protein can enhance the growth of *L. acidophilus*.

According to Zhao et al. [31] post-fermentation acidification had a negative effect on quality, and shortened the shelf life. Acid production development by *Bifidobacterium* is low in comparison to traditional yoghurt strains.

Several factors influence the viability of bacteria in yoghurt and other fermented dairy foods, in which the strains that are used and mutual interactions between species are of great importance. Low levels of *Bifidobacterium* in commercial yoghurts were correlated with high populations of *Lb. delbrueckii* subsp. *bulgaricus*, which produces acid rapidly and is responsible for an over-acidification of yoghurt at refrigeration temperatures. On the other hand, a stimulatory effect by *Lb. delbrueckii* subsp. *bulgaricus* on *B. bifidum* was also observed [6]. The acetic acid produced by *Bifidobacterium* could have a marginally inhibitory effect on *Lactobacillus* and *Streptococcus* spp. [6].

Our study showed that the addition of WPH to yoghurts preserved the viability of *Bifidobacterium adolescentis* co-incubated with yoghurt starter culture during fermentation. This is in accordance with reports by other authors [5,17,32]. The survival of probiotic bacteria was improved when milk was supplemented with casein and whey protein hydrolysates at concentrations ranging from 0.25 to 4 g/l [17]. A peptide hydrolysate from poultry bones and meat trimmings, obtained with commercially available enzymes (Alcalase 2.4 LFG, Neutrase 0.5 L, Protamex 1.5 MG, Flavorzyme 500MG), improved bacterial growth and maintained a high level of viability [13]. It was suggested that *Bifidobacterium* are either able to hydrolyse the peptides more efficiently than *Lactobacillus*, or that they find, among the peptides, some specific growth-promoting factors [13,20,26]. The human milk peptides that are known to exhibit growth-promoting effect on *Bifidobacterium* are characterized by the presence of one or two disulphide bonds within the single oligopeptide chain or between two different ones [14]. Enzymatic hydrolysis of whey proteins that are rich in cysteine residues could result in the formation of cystine-containing bifidogenic oligopeptides. Bovine whey proteins are mainly composed of β-lactoglobulin (BLG, 55-60 %) containing five cysteine residues. This protein is resistant to enzymatic hydrolysis in general. The enzyme used in this research, i.e. serine protease from *Y. lipolytica* offers the possibility of degrading the protein without needing additional technological treatments such as thermal or high pressure treatment [2].

CONCLUSION

This study suggests that the addition of WPH instead of SMP and WPC-80 in yoghurts may result in increased yoghurt culture counts at the initial stage of fermentation and significantly improve the viability of both yoghurt culture bacteria and *Bifidobacterium adolescentis*. In functional food application, the viability of probiotic strains is considered a measure for probiotic suitability. Moreover, the use of non-commercial serine protease from *Y. lipolytica* in the hydrolysis offers a promising low-cost alternative functional ingredient.

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The authors have no potential conflicts of interest to declare.