



University of Plovdiv
„Paisii Hilendarski”



Faculty of Biology
Department „Biochemistry and Microbiology”

TEODORA MINCHEVA PANAYOTOVA

INVESTIGATION OF THE PROPERTIES OF
BIOLOGICALLY ACTIVE PEPTIDES OBTAINED BY
ENZYMATIC HYDROLYSIS USING PROTEOLYTIC
ENZYMES FROM LACTIC ACID BACTERIA

ABSTRACT

of a dissertation
to acquire the educational and scientific degree „**doctor**”
Field of higher education: **4.** Natural sciences, mathematics and informatics
Professional direction: **4.3.** Biological sciences;
Doctoral program: **Biochemistry**

Supervisor: Prof. Ilia Nikolov Iliev, PhD

Plovdiv, 2026

The dissertation contains 182 pages, 9 tables, 52 figures, 2 appendices and 324 references.

All experimental work was done at the Department of "Biochemistry and Microbiology" at the Faculty of Biology, at the laboratories of Centre of technologies of the University of Plovdiv "Paisii Hilendarski" and at the laboratories of the R&D center "LB Bulgaricum" PLC, Sofia.

The PhD student has published a total of 3 articles in journals referenced and indexed in the databases Web of Science and Scopus.

The dissertation work was discussed and proposed for defense to the departmental council of the Department of "Biochemistry and Microbiology" at the Faculty of Biology of the PU "P. Hilendarski", held on March 10, 2026.

The open final meeting of the scientific jury will be held on June 9, 2026 at 11:00 in "Compass" hall of University of Plovdiv.

The materials on the defense are provided for free access of your interests in the library of PU "Paisii Hilendarski".

Author: Teodora Mincheva Panayotova

Title: Investigation of the properties of biologically active peptides obtained by enzymatic hydrolysis using proteolytic enzymes from lactic acid

Scientific jury:

Prof. Iskra Vitanova Ivanova, DBSc

Prof. Maria Bogomilova Angelova-Dyankova, DBSc

Prof. Penka Angelova Moncheva, PhD

Prof. Velizar Kostadinov Gochev, PhD

Assoc. prof. Tonka Atanasova Vasileva, PhD

Spare members:

Assoc. prof. Elena Dimitrova Apostolova-Kuzova, PhD

Assoc. prof. Nikolai Manchev Petrov, PhD

INTRODUCTION

In today's fast-paced world, food is regarded as a source of nutrients and bioactive compounds that improve human health and overall well-being. Growing consumer awareness of the impact of diet on health is reflected in their choice of natural products rich in vitamins, minerals, and other bioactive compounds, including bioactive peptides (Zaky A.A. et al. 2019; Zaky A.A. et al. 2020). Bioactive peptides are protein fragments that influence overall human health. Most bioactive peptides consist of two (dipeptides) to 20 amino acid residues and have a molecular weight of 0.4–2 kDa (Korhonen H. and Pihlanto A., 2006). In rare cases, longer peptides have also been reported. Bioactive peptides generated from protein-rich foods possess excellent potential for the creation of functional foods and/or dietary supplements for the prevention or treatment of certain chronic diseases such as cardiovascular diseases, diabetes, and cancer (Chakrabarti S. et al., 2014). They are considered a new generation of biologically active homeostasis regulators that have significant biological effects and exert a positive influence on the function or condition of the human body.

In recent years, new plant-based foods and beverages have been developed and introduced to the market to meet the growing demand for alternatives to animal-derived products. Milk and dairy products have long been regarded as a food group with a balanced composition that is essential for human nutrition.

However, people suffering from health issues such as lactose intolerance and milk protein allergies must consume alternative products. To enhance the health benefits of plant-based foods, new fermented products are being developed using plant proteins and lactic acid bacteria.

The breakdown of proteins by lactic acid bacteria plays a key role in the formation of bioactive peptides and amino acids, which are essential for bacterial growth and the production of metabolites that contribute to the taste and aroma of fermented products. Among the most studied bioactive peptides are ACE-inhibiting peptides, which have the ability to inhibit the activity of angiotensin-converting enzyme (ACE) and serve as an alternative to synthetic drugs for lowering blood pressure.

AIM AND TASKS

The aim of the present thesis is to investigate the production of bioactive peptides with ACE-inhibitory activity during the cultivation of lactic acid bacteria in media containing plant proteins.

To achieve the set goal, it is necessary to solve the following tasks:

1. Screening of *Lactobacillus* strains capable of hydrolyzing plant proteins during the fermentation process.
2. Optimize the conditions for the secretion of proteolytic enzymes by the selected *Lactobacillus* strains in the presence of various plant proteins.
3. Investigate the secretion of aminopeptidase enzymes by the selected *Lactobacillus* strains in the presence of various plant proteins.
4. To optimize the conditions for the proteolysis of plant proteins to obtain biologically active peptides with ACE-inhibitory activity from the selected *Lactobacillus* strains in the presence of various plant proteins.
5. To characterize the resulting bioactive peptides with ACE-inhibitory activity.

MATERIALS AND METHODS

1. Materials

1.1 *Lactobacillus* strains

Fifty strains of lactobacilli from four species were used in all experiments: *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus helveticus*, *Lacticaseibacillus casei*, and *Lactiplantibacillus plantarum* provided by the collection of “LB Bulgaricum” PLC, Sofia, Bulgaria.

1.2 Culture media

1.2.1 Culture media containing milk proteins

1.2.2 Culture media containing plant proteins

1.2.3 Culture media containing milk and plant proteins

1.2.4 Others

2. Methods

2.1 Cultivation conditions for lactobacilli

When testing proteolytic activity using the Church method, the strains *L. delbr.* ssp. *bulgaricus* and *L. helveticus* were initially cultured for 16 hours at 37°C and 42°C in sterile 10% reconstituted skim milk powder. *L. casei* and *L. plantarum* strains were tested after 16- and 24- hours of incubation at 32°C and 37°C in culture media consisting of sterile 10% reconstituted skim milk and sterile 10% reconstituted skim milk in the presence of 0,5% yeast extract. Following this screening, the selected *Lactobacillus* strains were cultured for 16 hours at 37°C in the respective culture media.

For all other methods, the culture media were inoculated with 1% of the selected *Lactobacillus* strains and cultured for 16 hours at 37°C.

2.2 Determination of degree of proteolysis by the OPA method (Church)

The analysis is based on reaction of o-phthaldialdehyde (OPA) and β -mercaptoethanol with primary amines to form 1-thioalkyl-2-alkylisoindoles, which absorb strongly at 340 nm. A standard curve using L-methionine was constructed and results presented as mmol/L methionine equivalents. Values for uninoculated media were subtracted to obtain final results.

2.3 Determination of proteolytic activity by Folin–Ciocalteu method

For this assay, selected *L. delbr.* ssp. *bulgaricus*, *L. helveticus* and *L. casei* strains were cultivated in media containing 10% skimmed milk powder, mMRS, mMRS + 1% pea protein hydrolysate, mMRS + 1% soy protein hydrolysate and mMRS + 1% whey protein WP80.

Casein was used as a substrate. Protease hydrolysis of casein releases tyrosine, quantifiable colorimetrically with Folin reagent. A standard curve with L-tyrosine was constructed.

2.4 Protein quantification by Bradford assay

This colorimetric method uses Coomassie Brilliant Blue G-250 dye binding to proteins, shifting absorbance from 465 nm to 595 nm. Cell lysates prepared similarly to those used in Folin–Ciocalteu assays were used for protein content determination.

2.5 Determination of aminopeptidase activity by the methods of Sasaki M. et al. and Eleman T.C.

Four synthetic substrates were used: L-leucine-p-nitroanilide (L-leu-pNA), L-lysine-p-nitroanilide (L-lys-pNA), L-arginine- β -naphthylamide (L-arg- β NA) and L-proline- β -naphthylamide (L-pro- β NA).

2.6 Determination of ACE-inhibitory activity by methods of Cushman, Cheung and Nakamura

The assay is based on the ability of a sample to block ACE activity. ACE is incubated with the substrate hippuryl-L-histidyl-leucine (HHL) and the formed product, hippuric acid, is measured spectrophotometrically.

2.7 Electrophoretic analysis by Tris–tricine SDS–PAGE (Schägger & von Jagow)

This method separates proteins and peptides in the range 1–100 kDa. Tricine enables separation of small proteins and peptides under 30 kDa at lower acrylamide concentrations.

2.8 Analysis of low-molecular-weight peptides by UHPLC–MS

Ultra-high-performance liquid chromatography–mass spectrometry (UHPLC–MS) identifies, sequences and characterizes low-molecular-weight peptides using sub- $2\ \mu\text{m}$ particle columns and higher pressures for faster analyses, improved resolution and greater sensitivity.

RESULTS AND DISCUSSION

1. Screening of lactic acid bacterial strains secreting proteolytic enzymes

1.1 Comparison of degree of proteolysis during cultivation of lactobacilli from different species

The degree of proteolysis of four lactobacillus species - *L. delbr. ssp. bulgaricus*, *L. helveticus*, *Lacticaseibacillus casei* and *Lactiplantibacillus plantarum* - was investigated in medium containing 10% skimmed milk powder (SMP). The OPA reagent method allows quantitative determination of amino acids released during proteolysis; background from the blank medium (uninoculated sample) was subtracted. L-methionine was the quantitative standard and results are presented as mmol methionine equivalents per liter (mmol/L). Results in Table 1 show strain-specific ability to proteolyze milk proteins in the tested medium. *L. delbr. ssp. bulgaricus* strains showed a wide range of accumulated proteolysis products at 37°C and 42°C (Table 1). At 37°C the highest amounts were observed for *L. bulgaricus* b283 and *L. bulgaricus* b81/1 (7,9 mmol/L), followed by *L. bulgaricus* b57/4 (7,7 mmol/L), *L. bulgaricus* b68/13 (7,6 mmol/L) and *L. bulgaricus* b77 (7,6 mmol/L). At 42°C higher values were generally observed. *L. bulgaricus* strain J24 showed 9,4 mmol/L methionine equivalents. For nearly one third of tested *L. bulgaricus* strains proteolysis was below 5,0 mmol/L at both temperatures, with relatively similar values. Low proteolysis at 42°C was noted for *L. bulgaricus* b123 and b263 — 1,9 mmol/L and 2,3 mmol/L, respectively. These results show *L. bulgaricus* strains exhibit a broad range of proteolytic capacity, allowing selection according to desired properties of the final product where they serve as starter cultures or in other fermentations. For *L. helveticus* strains, accumulated proteolysis products ranged from 5,9 mmol/L to 10.5 mmol/L at 37°C and from 6,1 mmol/L to 9 mmol/L at 42°C (Table 2). Results across the tested strains were relatively similar at both temperatures; as with *L. bulgaricus*, *L. helveticus* proteolysis was fairly consistent across temperatures.

Table 1. Degree of proteolysis results for *Lactobacillus delbr. ssp. bulgaricus* strains cultivated in 10% SMP at the 16th hour

Strains	37°C	42°C
<i>L. bulgaricus</i> b3/1	5,2 ± 0,1	6,8 ± 0,2
<i>L. bulgaricus</i> b8/2	6,4 ± 0,2	7,1 ± 0,2
<i>L. bulgaricus</i> 4/13	5,3 ± 0,1	7,8 ± 0,2
<i>L. bulgaricus</i> b5	4,2 ± 0,1	6,0 ± 0,1
<i>L. bulgaricus</i> b26	3,6 ± 0,1	4,7 ± 0,1
<i>L. bulgaricus</i> b27	7,1 ± 0,2	7,8 ± 0,2
<i>L. bulgaricus</i> b28	3,1 ± 0,1	2,9 ± 0,1
<i>L. bulgaricus</i> b37	4,6 ± 0,1	5,3 ± 0,2
<i>L. bulgaricus</i> b41	4,9 ± 0,1	2,8 ± 0,1
<i>L. bulgaricus</i> b54/7	5,8 ± 0,2	4,2 ± 0,1
<i>L. bulgaricus</i> b55/6	5,8 ± 0,2	7,8 ± 0,2
<i>L. bulgaricus</i> b56/7	6,7 ± 0,2	7,6 ± 0,2
<i>L. bulgaricus</i> b57/4	7,7 ± 0,2	8,8 ± 0,3
<i>L. bulgaricus</i> b58/1	6,0 ± 0,2	7,3 ± 0,2
<i>L. bulgaricus</i> b65/4	5,9 ± 0,1	7,0 ± 0,2
<i>L. bulgaricus</i> b67/2	6,0 ± 0,2	7,4 ± 0,2
<i>L. bulgaricus</i> b67/5	6,0 ± 0,2	8,5 ± 0,2
<i>L. bulgaricus</i> b68/13	7,6 ± 0,2	8,5 ± 0,2
<i>L. bulgaricus</i> b69/2	6,4 ± 0,2	8,5 ± 0,2
<i>L. bulgaricus</i> b69/4	6,2 ± 0,2	7,7 ± 0,2
<i>L. bulgaricus</i> b77	7,6 ± 0,2	7,8 ± 0,2
<i>L. bulgaricus</i> b120	4,0 ± 0,1	4,2 ± 0,1
<i>L. bulgaricus</i> b123	1,7 ± 0,1	1,9 ± 0,1
<i>L. bulgaricus</i> b144	3,7 ± 0,1	5,0 ± 0,2
<i>L. bulgaricus</i> b263	2,2 ± 0,1	2,3 ± 0,1
<i>L. bulgaricus</i> b283	7,9 ± 0,2	6,0 ± 0,2
<i>L. bulgaricus</i> bII3/2	3,3 ± 0,1	3,4 ± 0,1
<i>L. bulgaricus</i> b2380/8	4,0 ± 0,1	5,6 ± 0,2
<i>L. bulgaricus</i> b81/1	7,9 ± 0,2	7,6 ± 0,2
<i>L. bulgaricus</i> J24	5,0 ± 0,2	9,4 ± 0,3

* mmol methionine equivalents per liter ± SD from three replicates;

*blank medium value (5 mmol/L) subtracted

Table 2. Degree of proteolysis results for *Lactobacillus helveticus* strains cultivated in 10% SMP at the 16th hour

Strain	37°C	42°C
<i>L. helveticus</i> AB	6,9 ± 0,1	7,0 ± 0,1
<i>L. helveticus</i> 1/1	7,0 ± 0,2	8,6 ± 0,2
<i>L. helveticus</i> 7/6	7,6 ± 0,2	7,2 ± 0,1
<i>L. helveticus</i> 7/8	7,9 ± 0,2	7,3 ± 0,1
<i>L. helveticus</i> h9	7,3 ± 0,1	7,0 ± 0,1
<i>L. helveticus</i> h25	8,5 ± 0,2	7,2 ± 0,1
<i>L. helveticus</i> h48	9,0 ± 0,2	9,0 ± 0,2
<i>L. helveticus</i> h70	8,4 ± 0,2	7,6 ± 0,2
<i>L. helveticus</i> Q40	5,9 ± 0,1	6,1 ± 0,1
<i>L. helveticus</i> b230	10,5 ± 0,3	7,5 ± 0,1
<i>L. helveticus</i> b244	8,0 ± 0,2	6,4 ± 0,1

* mmol methionine equivalents per liter ± SD from three replicates;

*blank medium value (5 mmol/L) subtracted

In studies of proteolysis for *Lacticaseibacillus casei* strains incubated in 10% SMP, cultivation temperatures were 32°C and 37°C for 16 and 24 hours. No proteolysis was detected after 16 hours fermentation process at both temperature regimes. After 24 hours of cultivation in the medium, minimal amounts of proteolysis products were measured in the range 0.4–1.7 mmol/L with no difference between 32°C and 37°C (Table 3).

For strains of the species *Lactiplantibacillus plantarum*, no proteolysis was detected for any tested strain even after 24 hours of incubation in milk medium. This indicates that representatives of this species cannot degrade milk proteins, and therefore their growth in milk medium is limited.

Table 3. Degree of proteolysis for *Lacticaseibacillus casei* strains in 10% skimmed milk powder (SMP) at the 24th hour of cultivation

Strain	32°C	37°C
<i>L. casei</i> c 1	1,2 ± 0,1	1,7 ± 0,1
<i>L. casei</i> c 3	0,5 ± 0,05	0,5 ± 0,05
<i>L. casei</i> c 6	0,5 ± 0,05	0,4 ± 0,05
<i>L. casei</i> c 31	0,7 ± 0,05	0,5 ± 0,05

*mmol methionine equivalents per liter (mmol/L) ± SD from three replicates

*blank medium value (5 mmol/L) subtracted

1.2 Investigation of degree of proteolysis of lactobacillus strains cultivated in the presence of plant proteins

1.2.1. Cultivation of *Lactobacillus delbr. ssp. bulgaricus* strains in media with plant proteins

Based on obtained results for degree of proteolysis in 10% SMP, eight *L. delbr. ssp. bulgaricus* strains, five *L. helveticus* strains and three *Lacticaseibacillus casei* strains were selected for cultivation in media with plant proteins. The initial screening used four types of plant proteins — pea, soy, hemp and rice proteins.

Degree of proteolysis of strains grown in plant media was compared to that in 10% SMP and in a combined medium containing 10% SMP + 5% pea protein. Amounts of proteolysis products in plant media containing pea and soy protein were relatively close, ranging 1,3–4,3 mmol/L and 1,5–3,3 mmol/L, respectively. The highest degree of proteolysis in plant media was found in pea-protein medium for *L. bulgaricus* J24 – 4,3 mmol/L methionine equivalents (Fig. 1). With soy protein the highest proteolysis was for *L. bulgaricus* bLb81/1 – 3,3 mmol/L. Cultivation of *L. bulgaricus* strains in media with hemp and rice protein did not yield substantial amounts of proteolysis products.

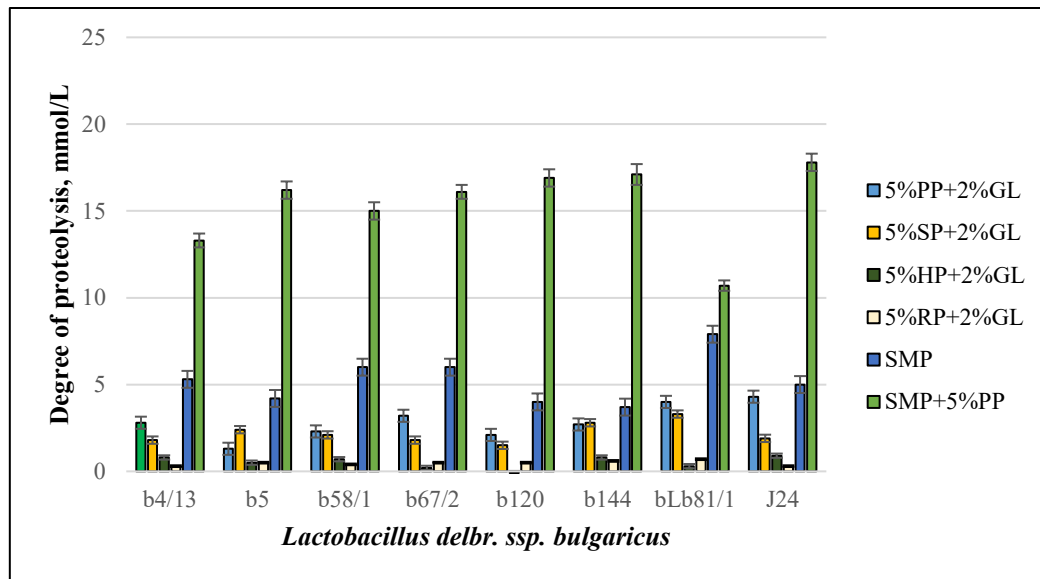


Figure 1. Degree of proteolysis of *Lactobacillus delbr. ssp. bulgaricus* strains in various plant media

*SMP — 10% skimmed milk powder; SMP + 5% PP — 10% SMP + pea protein; 5% PP + 2% GL — pea protein + glucose; 5% SP + 2% GL — soy protein + glucose; 5% HP + 2% GL — hemp protein + glucose; 5% RP + 2% GL — rice protein + glucose;

*mmol methionine equivalents per liter (mmol/L) ± standard deviation from three parallel measurements;

*Values have the uninoculated medium blank (5 mmol/L) subtracted.

1.2.2 Cultivation of *Lactobacillus helveticus* strains in media with plant proteins

L. helveticus strains showed a similar trend to *L. delbr. ssp. bulgaricus* with pronounced strain-specific proteolysis (Fig. 2). Amounts of proteolysis products in pea-protein medium ranged from 3,5 to 10,1 mmol/L. The highest proteolysis in pea medium was for *L. helveticus* b244 – 10,1 mmol/L methionine equivalents. With soy protein the highest proteolysis was again for *L. helveticus* b244 – 3,7 mmol/L. Cultivation of *L. helveticus* in hemp and rice protein media produced lower proteolysis amounts compared with other media, with values 1,4 – 2,3 mmol/L and 0,6 – 1,1 mmol/L, respectively.

The highest overall degree of proteolysis among all tested media was observed for *L. helveticus* strains cultivated in the combined medium 10% SMP + 5% pea protein (Fig. 2). The highest value was for *L. helveticus* b244 – 23,5 mmol/L methionine equivalents. Other strains had relatively close proteolysis values ranging 19,8–21,9 mmol/L.

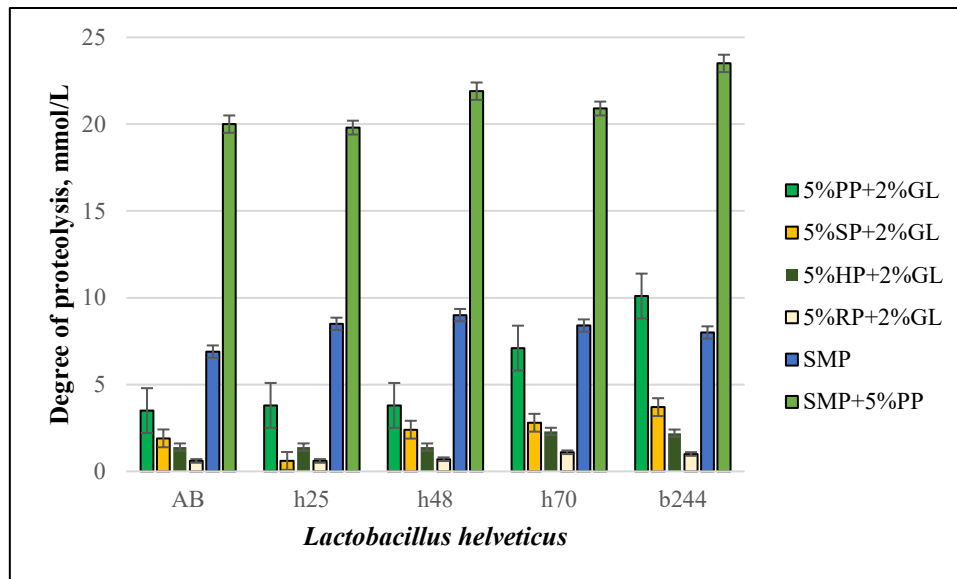


Figure 2. Degree of proteolysis of *L. helveticus* strains in various media

*SMP — 10% skimmed milk powder; SMP + 5% PP — 10% SMP + pea protein; 5% PP + 2% GL — pea protein + glucose; 5% SP + 2% GL — soy protein + glucose; 5% HP + 2% GL — hemp protein + glucose; 5% RP + 2% GL — rice protein + glucose *mmol methionine equivalents per liter (mmol/L) ± standard deviation from three parallel measurements; *Values have the uninoculated medium blank (5 mmol/L) subtracted.

1.2.3 Cultivation of *Lactobacillus casei* strains in media with plant proteins

Degree of proteolysis of three *L. casei* strains was studied in plant media. Amounts of proteolysis products in pea-protein medium ranged 1,8–5,6 mmol/L. The highest proteolysis in pea medium was for *L. casei* c1 — 5,6 mmol/L methionine equivalents. Moreover, *L. casei* c1 showed higher proteolysis compared with three of the tested *L. helveticus* strains and all *L. bulgaricus* strains in pea medium. Cultivation of *L. casei* in soy, hemp and rice protein media yielded lower proteolysis amounts: soy 0,6–1,9 mmol/L, hemp 0,1–0,5 mmol/L, rice 0,3–0,7 mmol/L, respectively.

The highest degree of proteolysis among all tested media was observed for *L. casei* strains cultivated in the combined medium 10% SMP + 5% pea protein (Fig. 3). The highest value was for *L. casei* c1 — 9,3 mmol/L methionine equivalents.

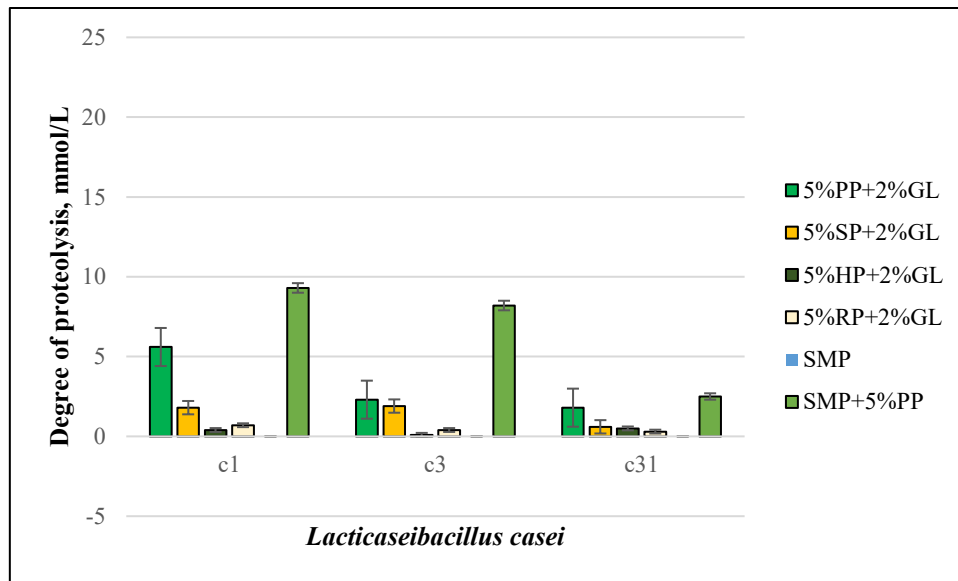


Figure 3. Degree of proteolysis of *L. casei* strains in various media

*SMP — 10% skimmed milk powder; SMP + 5% PP — 10% SMP + pea protein; 5% PP + 2% GL — pea protein + glucose; 5% SP + 2% GL — soy protein + glucose; 5% HP + 2% GL — hemp protein + glucose; 5% RP + 2% GL — rice protein + glucose.

(No proteolysis products were detected in SMP for these strains; results not shown in the figure).

*mmol methionine equivalents per liter (mmol/L) \pm standard deviation from three parallel measurements;

*Values have the uninoculated medium blank (5 mmol/L) subtracted.

1.3 Conclusion

The highest degree of proteolysis among the tested plant media was observed in media containing pea and soy protein. Experimental results showed that hemp and rice proteins are the least suitable substrates. Combining milk and pea protein activated proteolysis compared with other media, which is of interest for applying combined plant–milk media. *L. helveticus* strains metabolized plant media most intensively. After the study, strains with the highest proteolysis in plant media were identified as *L. helveticus* AB, *L. helveticus* h25, *L. helveticus* h48, *L. helveticus* h70, *L. helveticus* b244 and *L. casei* c1.

2. Investigation of specific proteolytic activity of lactobacillus strains cultivated in media with plant proteins

2.1 Comparative analysis of specific proteolytic activity in plant-protein media

Specific proteolytic activity was determined in pre-disrupted cells of selected strain *L. helveticus* AB, h25, h48, h70, b244 and *L. casei* c1 cultivated in 10% SMP, in mMRS (MRS without peptone and meat extract), mMRS + 1% pea protein hydrolysate, mMRS + 1% soy protein hydrolysate and mMRS + 1% whey protein (WP 80).

L. helveticus h70 showed the highest specific proteolytic activity in 10% SMP - 64,96 U/mg protein, followed by *L. helveticus* b244 - 35,81 U/mg protein and *L. helveticus* h25 – 13,63 U/mg protein. The tested *L. helveticus* strains had comparable or higher specific proteolytic activity values when different protein substrates were used.

The highest specific proteolytic activity in mMRS was for *L. helveticus* b244 – 57,29 U/mg protein. Similar values were measured for *L. helveticus* h70 (44,76 U/mg) and h25 (40,29 U/mg) (Fig. 4).

To assess the effect of 1% whey protein (WP 80) added to mMRS, WP80 activated the proteolytic system of *L. helveticus* b244, with protease activity comparable to that observed with 10% SMP and mMRS. For strains *L. helveticus* b244 and h70, the highest specific proteolytic activities measured were 53,71 U/mg and 30,26 U/mg protein, respectively (Fig. 4).

We tested the influence of different plant protein hydrolysates on specific proteolytic activity. Addition of 1% pea protein hydrolysate to mMRS increased specific proteolytic activity for *L. helveticus* AB and h48 to 18,51 U/mg protein and 19,38 U/mg protein, respectively (Fig. 4). The highest specific proteolytic activity in this medium was for *L. helveticus* b244 – 35,55 U/mg protein. *L. helveticus* b244 showed specific proteolytic activity comparable to that in milk-protein medium. Thus, addition of 1% pea protein hydrolysate to mMRS is a strain-specific activator of proteolytic activity in various lactobacillus strains.

Addition of 1% soy protein hydrolysate to mMRS showed a similar trend; however, specific proteolytic activity of *L. helveticus* strains was not strongly activated by soy hydrolysate. The highest and similar values were for *L. helveticus* h70 (17,90 U/mg protein) and b244 (16,85 U/mg protein) (Fig. 4).

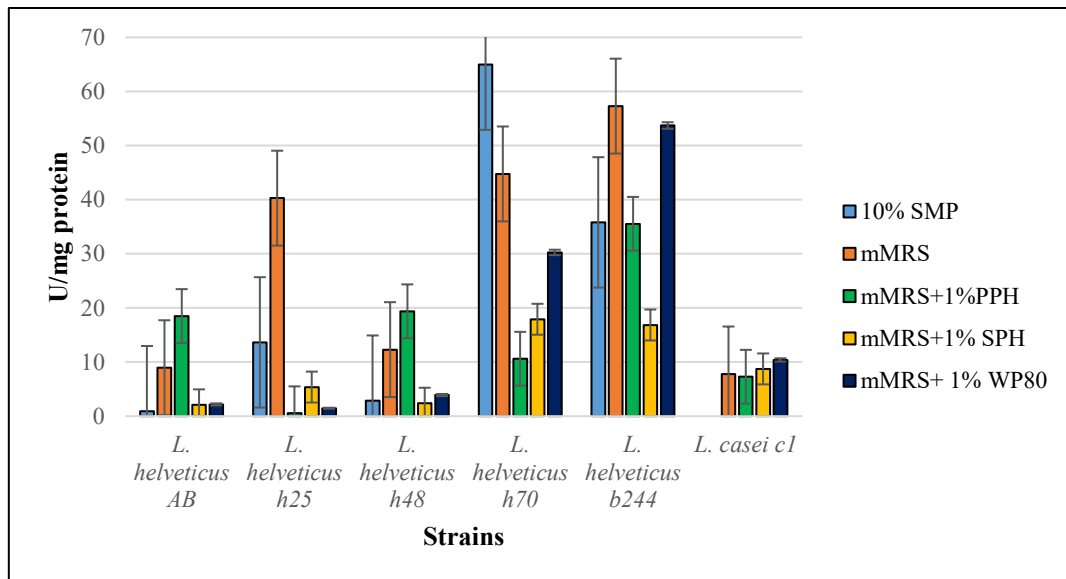


Figure 4. Specific proteolytic activity of lactobacillus strains in different media

*10% SMP — skimmed milk powder; mMRS — MRS without peptone and meat extract; PPH — pea protein hydrolysate; SPH — soy protein hydrolysate; WP 80 — whey protein 80; *Specific proteolytic activity \pm standard deviation from three parallel measurements

Compared with *L. helveticus* strains, *L. casei* c1 showed low specific proteolytic activity across all tested mMRS-based media. In mMRS without plant hydrolysates the strain showed activity similar to *L. helveticus* AB and h48 (7,80 U/mg protein). With 1% pea protein hydrolysate added to mMRS, activity (7,28 U/mg protein) approached that of *L. helveticus* h70 (10,61 U/mg protein). Activity in mMRS with soy hydrolysate was comparable to pea hydrolysate - 8.73 U/mg protein. Addition of WP 80 also had no substantial effect: 8,73 U/mg protein.

2.2 Conclusion

The highest specific proteolytic activities were found in 10% SMP and in mMRS. Addition of pea protein hydrolysate to mMRS increased specific proteolytic activity for *L. helveticus* AB and h48. The highest specific proteolytic activity in mMRS with pea hydrolysate was for *L. helveticus* b244, comparable to that in milk-protein medium. Specific proteolytic activity of the tested strains was not strongly activated by soy hydrolysate. Addition of WP 80 to mMRS activated proteolysis in *L. helveticus* b244, giving activity comparable to 10% SMP and mMRS. Pea and soy protein hydrolysates and whey protein WP 80 act as strain-specific activators of proteolytic activity in selected lactobacilli. *L. helveticus* b244 and h70 showed the highest specific proteolytic activities among tested media.

3. Investigation of aminopeptidase activity of selected lactobacillus strains cultivated in media with plant proteins

3.1 Investigation of leucine aminopeptidase and lysine aminopeptidase

Leucine and lysine aminopeptidase activities were determined in pre-disrupted cells of selected strains *L. helveticus* AB, h25, h48, h70, b244 and *L. casei* c1 cultivated in 10% SMP, mMRS (without peptone and meat extract), mMRS + 1% pea protein hydrolysate, mMRS + 1% soy protein hydrolysate and mMRS + 1% whey protein (WP 80).

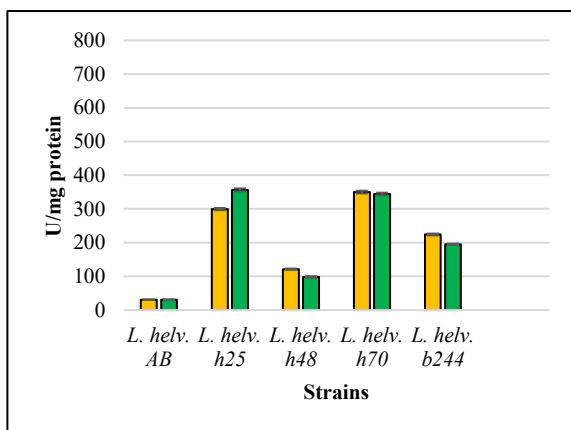
Tested lactobacillus strains showed variable activity against the substrates depending on medium. In 10% SMP, the highest specific leucine aminopeptidase activity was for *L. helveticus* h70 — 350 U/mg protein. A similar value was found for *L. helveticus* h25 — 299 U/mg. Similar results were obtained for lysine aminopeptidase in 10% SMP. *L. helveticus* h25 had the highest specific lysine aminopeptidase activity (356 U/mg protein), and *L. helveticus* h70 had similar values (344 U/mg protein) (Fig. 5). Milk-protein medium was unsuitable for secretion of aminopeptidases in *L. casei* c1.

Results for the two aminopeptidases in strains cultivated in mMRS were higher than those obtained in 10% SMP and across all tested media (Fig. 5B). *L. helveticus* h25 showed the highest specific leucine aminopeptidase activity - 696 U/mg protein. Other *L. helveticus* strains had relatively close specific leucine aminopeptidase activities ranging 155,42–307,90 U/mg protein. A similar trend was observed for lysine aminopeptidase activity. *L. helveticus* h25 again had the highest specific lysine aminopeptidase activity - 816 U/mg protein. Other *L. helveticus* strains had specific aminopeptidase activities in the range 207,23–488,40 U/mg protein. Specific lysine aminopeptidase activity for *L. casei* c1 was similar to its leucine aminopeptidase activity (84,77 U/mg).

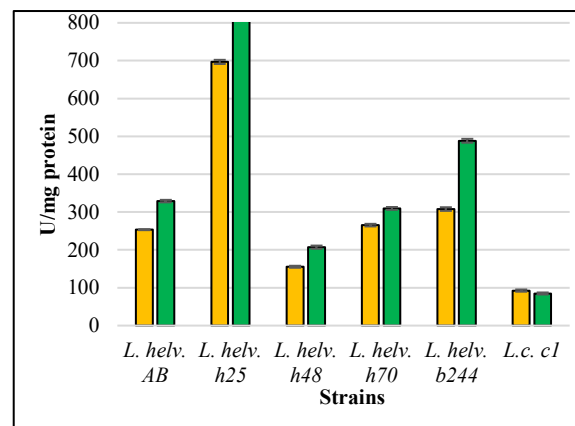
Addition of plant protein hydrolysates to mMRS caused a marked reduction of leucine and lysine aminopeptidase activities (Fig. 5 C, D). *L. helveticus* AB, h48, h70 and b244 strains showed similar leucine aminopeptidase activities in mMRS with pea hydrolysate, with values 98,51–119,44 U/mg protein (Fig. 5C). Specific leucine aminopeptidase activity of *L. helveticus* h25 in mMRS with pea hydrolysate was lowest but comparable to *L. helveticus* AB in 10% SMP (Fig. 5 A, C). Strains *L. helveticus* AB, h48, h70 and b244 showed comparable lysine aminopeptidase activities ranging 132,99–189.71 U/mg protein (Fig. 5C). Strains *L. helveticus* h25 and *L. casei* c1 showed comparable specific lysine and leucine aminopeptidase activities.

Addition of 1% soy protein hydrolysate to mMRS did not activate leucine and lysine aminopeptidases. Only *L. helveticus* AB showed leucine and lysine aminopeptidase activities comparable to values in mMRS without supplements. Specific leucine aminopeptidase activity for *L. helveticus* AB was 186,15 U/mg protein and specific lysine aminopeptidase activity was 246,91 U/mg protein (Fig. 5D). These results were similar to the values obtained in mMRS medium without supplements. Compared to the medium with 10% skimmed milk powder they were 6–7 times higher, and in the mMRS medium containing pea protein hydrolysate they were about 2 times higher. The two aminopeptidase activities for *L. helveticus* h70 strain were identical (159,26 U/mg protein) in mMRS with soy protein hydrolysate, close to the aminopeptidase activities in mMRS with pea protein hydrolysate and about two times lower compared to the medium with 10% skimmed milk powder and to mMRS without supplements.

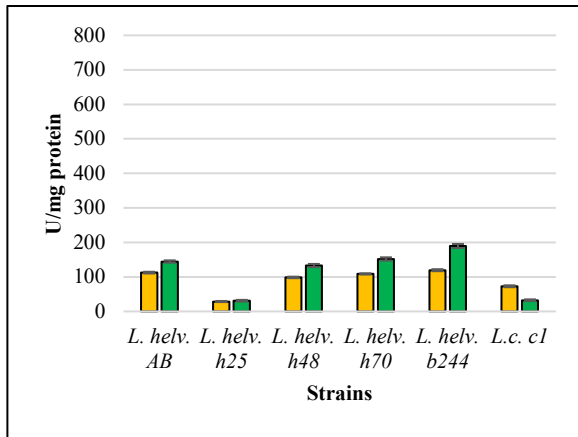
Addition of whey protein WP 80 to mMRS did not activate the proteolytic system in the tested lactobacillus strains (Fig. 5E). The highest specific leucine aminopeptidase activity was found for strain *L. helveticus* b244 – 222,96 U/mg protein. This value was comparable to results obtained in medium with 10% skimmed milk powder, and higher than the leucine aminopeptidase activities measured in media containing plant protein hydrolysates.



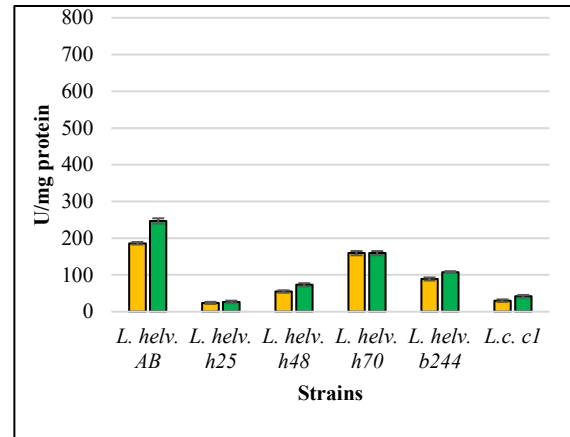
A. (10 % SMP)



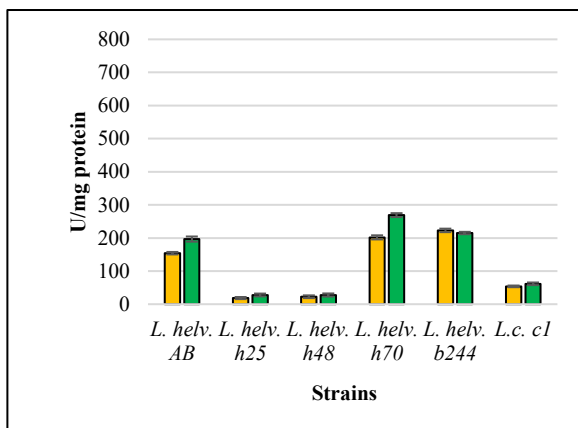
B. (mMRS)



C. (mMRS + 1% PPH)



D. (mMRS + 1% SPH)



E. (mMRS + 1% WP80)

Figure 5 (A–D). Specific leucine (■) and lysine (■) aminopeptidase activity of *L. helveticus* strains and *L. casei* c1 in different media

*10% SMP – 10% skimmed milk powder; mMRS — MRS without peptone and meat extract; PPH – pea protein hydrolysate; SPH – soy protein hydrolysate; WP 80 – whey protein 80; *L. helv.* – *L. helveticus*; *L.c. c1* – *L. casei* c1.

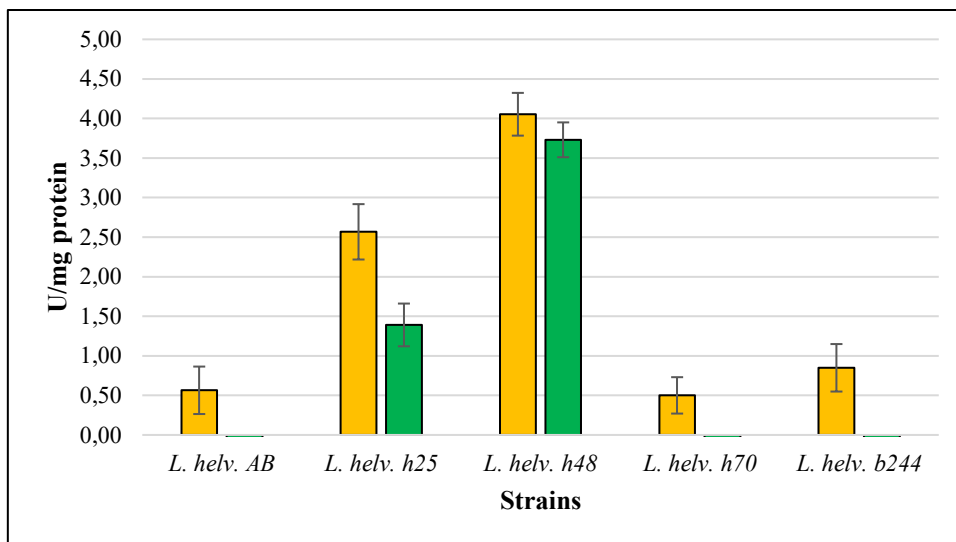
*Specific leucine and lysine aminopeptidase activity with standard deviation from three parallel measurements

*No specific leucine and lysine aminopeptidase activity detected in 10% SMP for strain *L. casei* c1

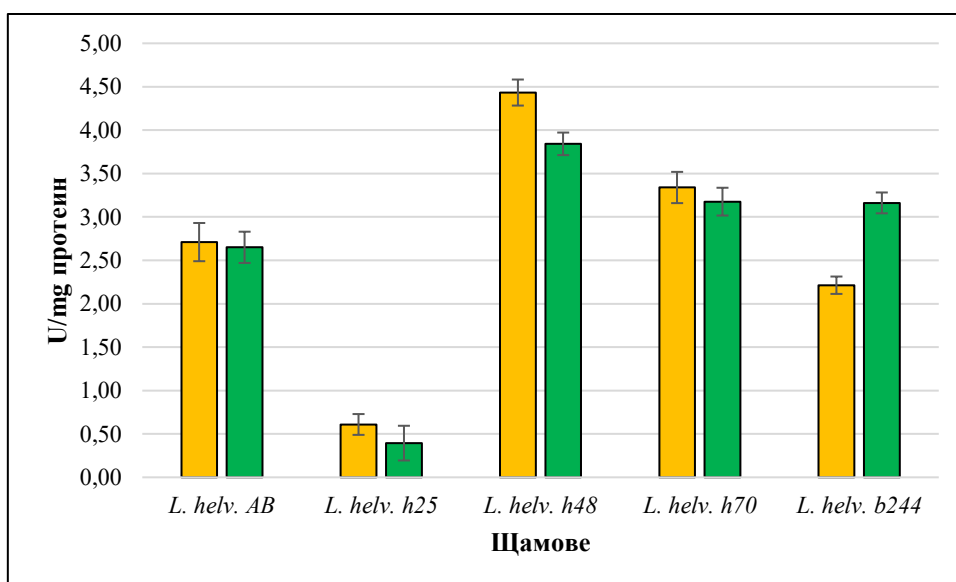
L. helveticus h70 and *L. helveticus* AB strains had leucine aminopeptidase activities similar to *L. helveticus* b244, with values of 201,88 U/mg protein and 154,12 U/mg protein, respectively. The highest specific lysine aminopeptidase activity was found for strain *L. helveticus* h70 - 269,17 U/mg protein. This value was higher compared with lysine aminopeptidase activities in media with plant protein hydrolysates (Fig. 5B, C, D). Strain *L. helveticus* b244 and strain *L. helveticus* AB showed comparable specific lysine aminopeptidase activities — 215 U/mg protein and 196,76 U/mg protein, respectively.

3.2. Investigation of arginine aminopeptidase and proline aminopeptidase activity

The specific arginine and proline aminopeptidase activities of the tested lactobacillus strains were significantly lower compared to the specific leucine and lysine aminopeptidase activities in all tested media (Fig. 6 A–B). Moreover, both activities were detected in 10% skimmed milk powder medium and in mMRS with 1% pea protein hydrolysate. In the milk-protein-containing medium with higher activity, arginine aminopeptidase was detected in all *L. helveticus* strains. Strains *L. helveticus* h48 and *L. helveticus* h25 showed the highest specific arginine aminopeptidase activities, with values of 4.05 U/mg protein and 2.57 U/mg protein. In the other *L. helveticus* strains the specific arginine aminopeptidase activity was below 1 U/mg protein (Fig. 6A). Specific proline aminopeptidase activity was measured only in strains *L. helveticus* h48 and *L. helveticus* h25, with values of 3,73 U/mg protein and 1,39 U/mg protein, respectively (Fig. 6A). Addition of 1% pea protein hydrolysate to mMRS led to activation of arginine and proline aminopeptidases in *L. helveticus* strains. The highest specific arginine aminopeptidase activity was found for *L. helveticus* h48 – 4,43 U/mg protein. Strains *L. helveticus* AB, *L. helveticus* h70 and *L. helveticus* b244 had similar specific arginine aminopeptidase activities – 2,71 U/mg protein, 3,34 U/mg protein and 2,21 U/mg protein, respectively. Values for specific proline aminopeptidase activity were comparable to the arginine aminopeptidase values. Again, *L. helveticus* h48 showed the highest specific proline aminopeptidase activity – 3,84 U/mg protein. *L. helveticus* AB, *L. helveticus* h70 and *L. helveticus* b244 strains exhibited comparable specific proline aminopeptidase activities – 2,65 U/mg protein, 3,18 U/mg protein and 3,16 U/mg protein, respectively. No arginine or proline aminopeptidase activities were detected for *L. casei* c1 in any tested media.



A. (10% SMP)



B. (mMRS + 1% PPH)

Figure 6 (A–B). Specific arginine (■) and proline (■) aminopeptidase activity of *L. helveticus* strains in different media

*10% SMP – 10% skimmed milk powder; mMRS — MRS without peptone and meat extract; PPH – pea protein hydrolysate; *L. helv.* – *L. helveticus*.

*Specific arginine and proline aminopeptidase activity with standard deviation from three parallel measurements

*No specific arginine and proline aminopeptidase activity detected in the tested media for strain *L. casei* c1.

3.3. Conclusion

The study showed that *L. helveticus* h25 strain had the highest specific leucine and lysine aminopeptidase activities obtained in mMRS. Addition of pea protein hydrolysate, soy protein hydrolysate and whey protein WP 80 did not activate leucine and lysine aminopeptidases in the tested lactobacillus strains. The specific arginine and proline aminopeptidase activities of the tested strains were significantly lower compared with the specific leucine and lysine activities in all tested media. Arginine and proline aminopeptidase activities were detected when *L. helveticus* strains were cultivated in 10% skimmed milk powder and in mMRS with 1% pea protein hydrolysate. Strain *L. helveticus* h48 showed the highest specific arginine and proline aminopeptidase activities among the tested media.

4. Demonstration of bioactive peptides in media with plant proteins

4.1. Investigation of ACE-inhibitory activity

The ACE-inhibitory activity of peptides obtained after cultivation of the pre-selected *L. helveticus* AB, *L. helveticus* h25, *L. helveticus* h48, *L. helveticus* h70, *L. helveticus* b244 and *L. casei* c1 strains was examined in 9% skimmed milk powder (SMP), 9% SMP with 5% pea protein, 9% SMP with 5% soy protein, a medium with 5% pea protein with 2% glucose, and a medium with 5% soy protein with 2% glucose. ACE-inhibitory activity showed strain-dependent specificity depending on the media used (Fig. 7). *L. helveticus* strains exhibited ACE-inhibitory activity ranging from 9 U/mg protein to 75,5 U/mg protein when cultivated in different media. Only strain *L. casei* c1 showed the lowest values. ACE-inhibitory activity of the tested lactobacilli cultivated in 9% SMP ranged from 23 U/mg protein to 54 U/mg protein. Strain *L. helveticus* b244 showed the highest ACE-inhibitory activity - 54 U/mg protein.

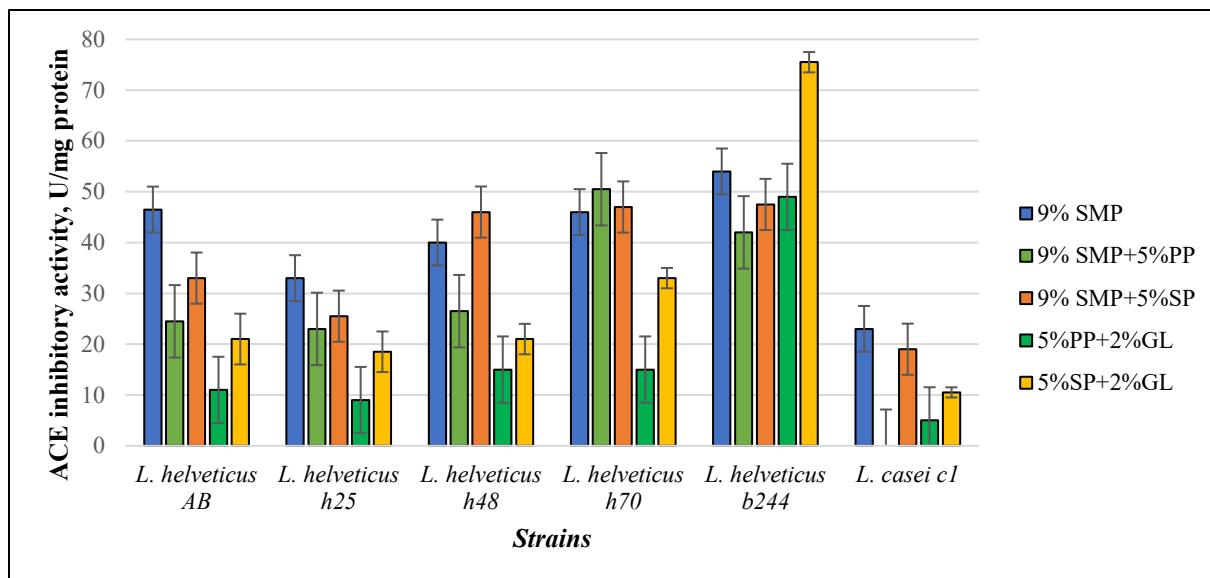


Figure 7. ACE-inhibitory activity of *L. helveticus* AB, *L. helveticus* h25, *L. helveticus* h48, *L. helveticus* h70, *L. helveticus* b244 and *L. casei* c1 cultivated in media with different protein sources

*9% SMP – 9% skimmed milk powder; 9% SMP + 5% PP – 9% SMP with added pea protein; 9% SMP + 5% SP – 9% SMP with added soy protein; 5% PP + 2% GL – pea protein with added glucose; 5% SP + 2% GL – soy protein with added glucose.

*ACE-inhibitory activity with standard deviation from three parallel measurements

*One unit of inhibitory activity (1 Unit) is defined as the amount of substance in 1 mg of supernatant from the tested culture that causes 50% inhibition (IC₅₀) of ACE.

We examined the influence of pea and soy protein in plant-based media on ACE-inhibitory activity of selected lactobacilli (Fig. 7). *L. helveticus* b244 showed the highest ACE-inhibitory activity in the plant medium containing 5% soy protein and 2% glucose — 75,5 U/mg protein. This value was about 40% higher than the ACE-inhibitory activity in the milk-only medium and about 60% higher compared to other media. ACE-inhibitory activity in the plant medium with soy protein was higher than in the plant medium with pea protein for all tested lactobacilli. Addition of pea and soy protein to the milk medium did not significantly affect ACE-inhibitory activity compared to 9% SMP. Combining 5% pea protein with 9% SMP resulted in about a 10% increase in ACE-inhibitory activity for *L. helveticus* h70. For the other *L. helveticus* strains pea protein had no effect. The highest ACE-inhibitory activity in the combined medium (9% milk + 5% pea) was observed for *L. helveticus* h70, with 50,5 U/mg protein. *L. helveticus* b244 showed a comparable ACE-inhibitory activity - 42 U/mg protein. Other *L. helveticus* strains had similar ACE-inhibitory activities between 23 U/mg protein and 26,5 U/mg protein. No ACE-inhibitory activity for *L. casei* c1 was detected in the combined

9% milk + 5% pea protein medium. Adding 5% soy protein to 9% SMP increased ACE-inhibitory activity of *L. helveticus* h48 by about 15%, reaching 46 U/mg protein. The ACE-inhibitory activity of *L. helveticus* h70 in 9% SMP + 5% soy protein was comparable to that in the milk-only medium — 47 U/mg protein.

4.2. Conclusion

L. helveticus b244 strain showed the highest ACE-inhibitory activity when cultivated in plant media containing 5% soy protein with 2% glucose and 5% pea protein with 2% glucose. Additionally, ACE-inhibitory activity was detected when cultivating combined media containing 9% milk protein with 5% pea protein and 9% milk protein with 5% soy protein for *L. helveticus* h70 and *L. helveticus* b244 strains. The highest ACE-inhibitory activity was obtained in plant medium consisting of 5% soy protein and 2% glucose. *L. helveticus* b244 showed the highest ACE-inhibitory values in all tested media.

5. Characterization of formed peptides

5.1. SDS-PAGE (Tris-tricine) electrophoresis of selected lactobacillus strains

SDS-PAGE (Tris-tricine) electrophoresis was performed on peptide fractions obtained from cultivation of strains *L. helveticus* h70 and *L. helveticus* b244 in various media containing plant proteins.

In 9% SMP, except for the hard to degrade casein complex at 38 kDa, all other protein bands were hydrolyzed in fermented samples by both *L. helveticus* strains. Some casein fractions were hydrolyzed during fermentation and the protein band at ~30 kDa was completely absent in fermented samples with *L. helveticus* strains. Proteolysis was also observed for β -lactoglobulin and α -lactalbumin fractions. As a result of proteolysis of high-molecular-weight peptides, new peptides of about 12 kDa appeared in the fermented samples with *L. helveticus* h70 and *L. helveticus* b244 that were not present in the uninoculated sample.

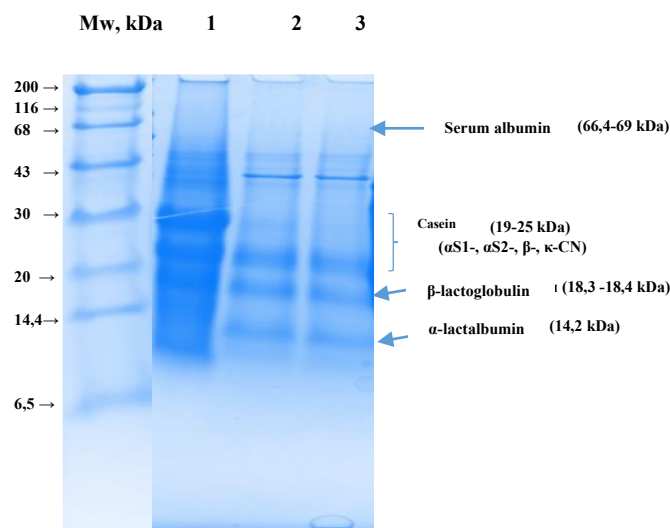


Figure 8. SDS-PAGE of *L. helveticus* h70 and *L. helveticus* b244 cultivated in 9% skimmed milk powder (SMP)

*1. 9% SMP, uninoculated medium; 2. *L. helveticus* h70 cultivated in 9% SMP; 3. *L. helveticus* b244 cultivated in 9% SMP.

Analysis of SDS-PAGE data for protein fractions of *L. helveticus* h70 and *L. helveticus* b244 strains cultivated in plant medium containing 5% pea protein with 2% glucose showed presence of new low-molecular-weight peptides (Fig. 8). Strongly stained bands were observed in the uninoculated medium, confirming the high molecular weight of pea proteins, whose protein composition ranges from 30 to 400 kDa (Yang J. et al., 2023). Milk protein fractions range 19–25 kDa (Elzoghby A.O. et al., 2015). Hydrolysis of vicilin subunits (~40 kDa) was observed. The staining intensity of the band in the uninoculated sample decreased compared to fermented samples with *L. helveticus* h70 and b244.

A similar trend was observed for protein bands in the 14,4–30 kDa range. Low-molecular-weight peptides were evident in fermented samples below 6,5 kDa, while none of these low-MW peptides were found in the uninoculated medium. The lower staining intensity of bands was almost undetectable after hydrolysis, likely due to further degradation to amino acids.

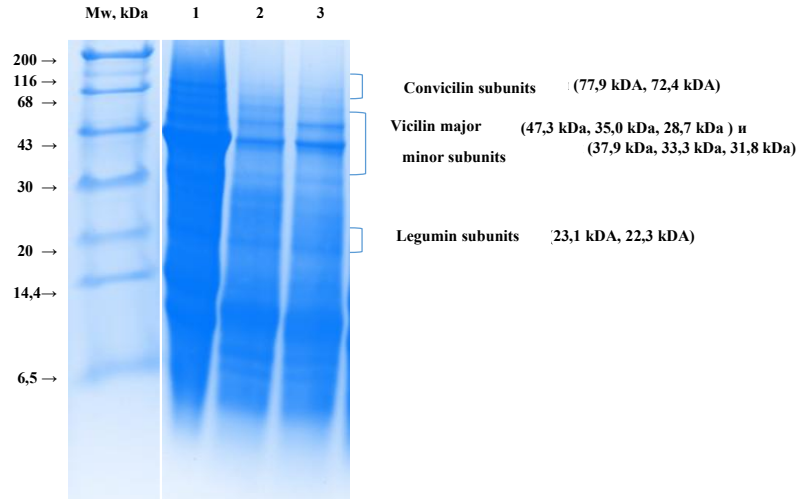


Figure 9. SDS-PAGE of *L. helveticus* h70 and *L. helveticus* b244 cultivated in plant medium 5% pea protein + 2% glucose

*1. 5% pea protein + 2% glucose, uninoculated medium; 2. *L. helveticus* h70 cultivated in 5% pea protein + 2% glucose; 3. *L. helveticus* b244 cultivated in 5% pea protein + 2% glucose.

Electrophoretic analysis confirmed that when *L. helveticus* h70 and b244 are cultivated in plant medium with 5% pea protein with 2% glucose, low-molecular-weight peptides (< 6.5 kDa) are formed, and vicilin hydrolysis is faster than legumin subunit hydrolysis. Also, the number of peptide bands under legumin subunit fractions is higher, indicating formation of lower-MW peptides.

In contrast to 9% SMP and the plant medium with 5% pea protein with 2% glucose, in the plant medium with 5% soy protein with 2% glucose no high-molecular-weight protein fractions above 30 kDa were detected either in uninoculated medium or fermented samples. Figure 10 shows hydrolysis of 11S glycinin with decreased staining intensity of the 27 kDa band in samples fermented with *L. helveticus* h70 and b244. Higher hydrolysis leads to lighter bands compared to the uninoculated sample. After fermentation in 5% soy protein with 2% glucose, 11S glycinin fractions were degraded to lower-MW fractions, producing new peptides around 12 kDa that were absent in the uninoculated sample. Hydrolysis of the acidic subunit of 11S glycinin occurred faster than that of the basic subunit. The number of peptide bands under the basic subunit fractions was higher, indicating formation of lower-MW peptides — important for reducing product allergenicity. SDS-PAGE results for fermented plant medium with 5% soy

protein with 2% glucose using *L. helveticus* h70 and b244 showed a lower degree of protein hydrolysis compared to fermented plant medium with 5% pea protein with 2% glucose.

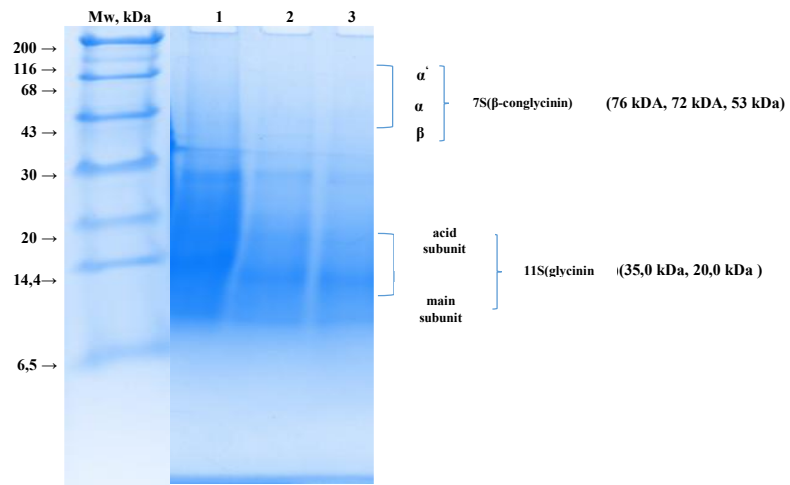


Figure 10. SDS-PAGE of *L. helveticus* h70 and *L. helveticus* b244 cultivated in plant medium 5% soy protein + 2% glucose

*1. 5% soy protein + 2% glucose, uninoculated medium; 2. *L. helveticus* h70 cultivated in 5% soy protein + 2% glucose; 3. *L. helveticus* b244 cultivated in 5% soy protein + 2% glucose.

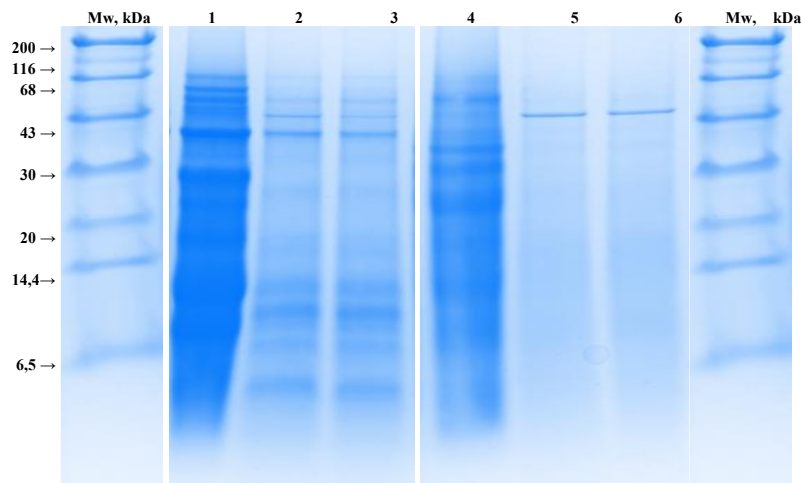


Figure 11. SDS-PAGE of *L. helveticus* h70 and *L. helveticus* b244 cultivated in combined media

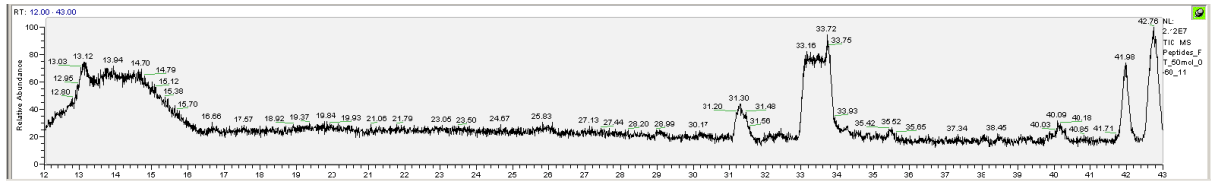
*1. 9% SMP + 5% pea protein, uninoculated; 2. *L. helveticus* h70 cultivated in combined 9% SMP + 5% pea; 3. *L. helveticus* b244 cultivated in combined 9% SMP + 5% pea; 4. 9% SMP + 5% soy protein, uninoculated; 5. *L. helveticus* h70 cultivated in combined 9% SMP + 5% soy; 6. *L. helveticus* b244 cultivated in combined 9% SMP + 5% soy. In combined media a similar proteolysis trend during fermentation by *L. helveticus* h70 and b244 was observed, with significant reduction in staining intensity of protein bands compared to uninoculated medium. The results clearly demonstrate the proteolytic capability of *L. helveticus* h70 and b244 to degrade not only milk proteins but also protein fractions in media containing pea and soy proteins to varying degrees.

Similar tendency was observed in presented combined media fermented with *L. helveticus* h70 и *L. helveticus* b244 strains. The obtained results demonstrate proteolysis of *L. helveticus* h70 и *L. helveticus* b244 strains in milk and plant media.

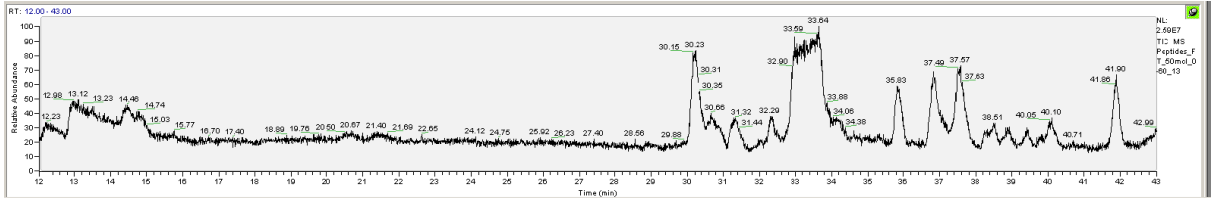
5.2. Detection of newly formed peptide fractions by ultra-high-performance liquid chromatography–mass spectrometry (UHPLC-MS)

Preselected strains *L. helveticus* h70 and *L. helveticus* b244 were cultivated in 9% SMP, 9% SMP with 5% pea protein, 9% SMP with 5% soy protein, 5% pea protein with 2% glucose and 5% soy protein with 2% glucose. Peptide profiles of *L. helveticus* h70 and b244 in milk medium are shown in Fig. 12. The uninoculated sample (9% SMP) (Fig. 12A) was characterized by four main peptide fractions with retention times 31,30 min, 33,16–33,75 min, 41,98 min and 42,76 min. These fractions were easily recognized in samples fermented by both strains except the fourth at 42,76 min. Fermentation by both *L. helveticus* strains led to detection of two new peaks in the 30,20–30,66 min range before the first major fraction. The first new peak at 30,23 min was more pronounced in *L. helveticus* h70 (Fig. 12B) compared to *L. helveticus* b244 (Fig. 12 C). The second new peptide fraction (30,66 min) was relatively similar for both strains. After the first main fraction (31,30 min) another similar peak appeared for both strains at 32,28–32,29 min. Unlike the first major fraction, the second showed multiple peaks for both *L. helveticus* strains. The third major peptide fraction remained almost unchanged after lactic acid fermentation, while the last fraction was degraded by the proteolytic systems of *L. helveticus* h70 and b244 and was completely absent.

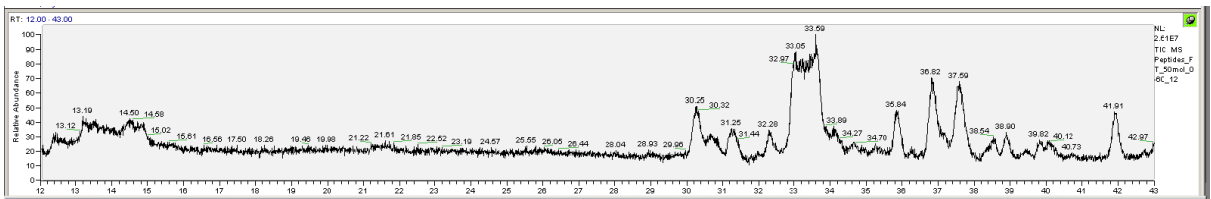
The uninoculated sample (5% pea protein with 2% glucose) (Fig. 13A) was characterized by three main peptide fractions: high- (30.71 min), medium- (34.60 min) and low- (43.25 min) molecular weight peptide fractions. For both tested strains, the high- and medium-MW peptide fractions were completely degraded in fermented samples, with appearance of many new peptide fractions (Fig. 13B, C). Fermented samples contained many low-MW peptides in the 36–42 min retention time range; peaks after 41 min were stronger, indicating greater degradation to low-MW peptides. Fig. 13 results show that proteins and higher-MW peptides were degraded to lower-MW peptides during fermentation. For both *L. helveticus* strains no significant differences were observed in the degree of degradation and appearance of new peaks. Slightly more peaks in 35–41 min retention time range were observed in samples with *L. helveticus* b244 compared to h70.



A



B

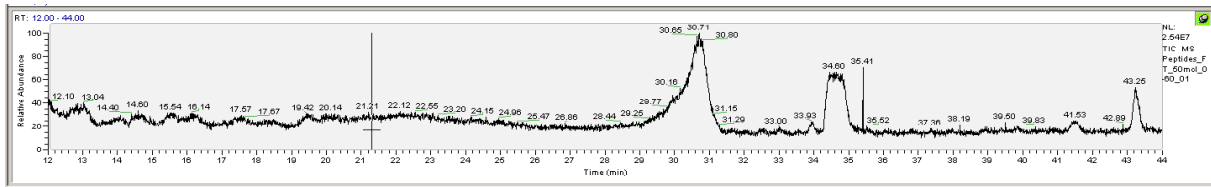


C

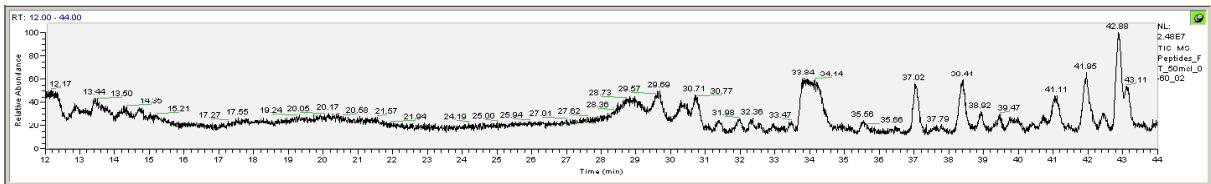
Figure 12. (A–C) Peptide profiles obtained from cultivation of *L. helveticus* h70 and b244 in 9% SMP

A. 9% SMP, uninoculated; B. h70 in 9% SMP; C. b244 in 9% SMP.

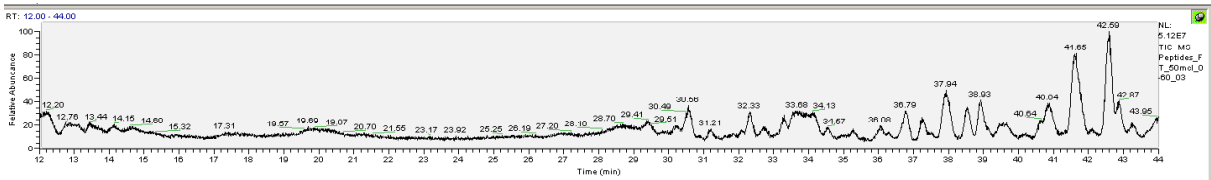
*9% SMP – 9% skimmed milk powder



A



B



C

Figure 13. (A–C) Peptide profiles obtained from cultivation of *L. helveticus* h70 and b244 in 5% pea protein + 2% glucose

A. 5% pea protein + 2% glucose, uninoculated; B. h70 in 5% pea protein + 2% glucose; C. b244 in 5% pea protein + 2% glucose.

In contrast to profiles from plant medium with 5% pea protein with 2% glucose fermented with *L. helveticus* h70 and b244, the plant medium containing 5% soy protein with 2% glucose did not show significant degradation of peptide fractions (Fig. 14). The uninoculated sample (5% soy protein with 2% glucose) (Fig. 14A) had two main peptide fractions at 14,46 min and 23,27 min. These fractions were also present in fermented samples from both strains. Numerous secondary peaks between these two main peaks were observed in both uninoculated and fermented samples. These results suggest that soy proteins and higher-MW peptides are more difficult to degrade, or are not degraded to lower-MW peptides during fermentation.

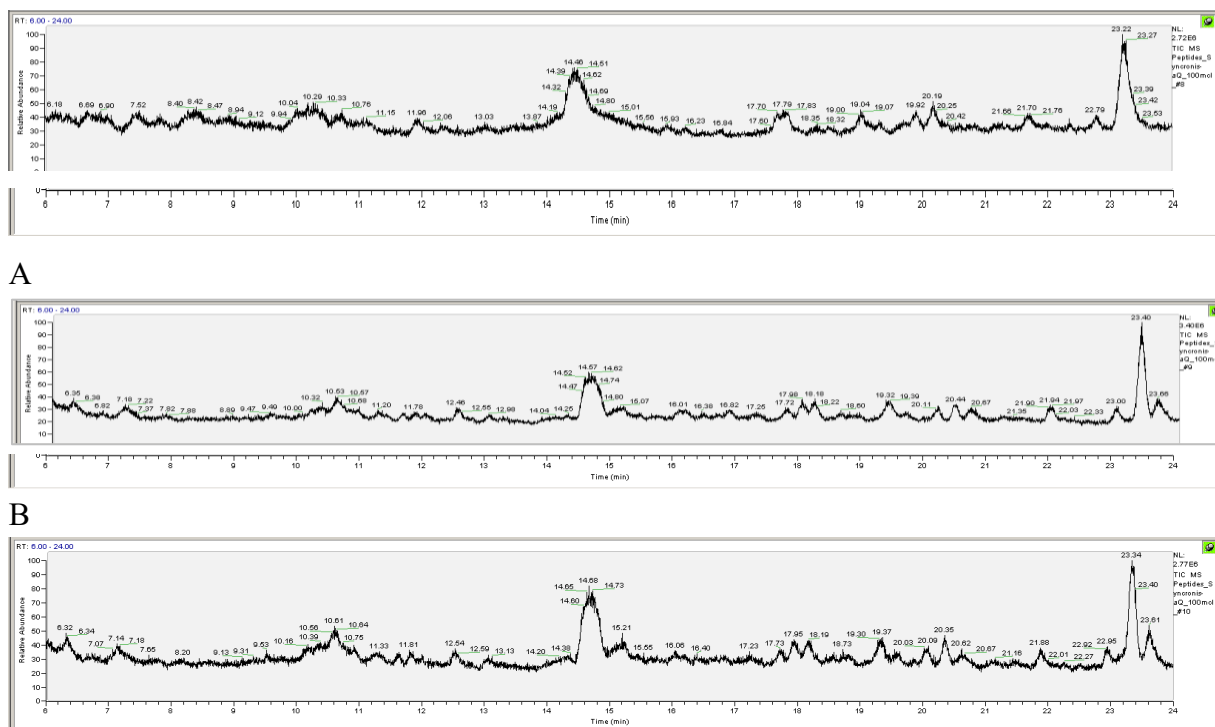


Figure 14. (A–C) Peptide profiles obtained from cultivation of *L. helveticus* h70 and b244 in 5% soy protein + 2% glucose

A. 5% soy protein + 2% glucose, uninoculated; B. h70 in 5% soy protein + 2% glucose; C. b244 in 5%

5.3 Conclusion

Following SDS-PAGE electrophoresis and UHPLC-MS analysis, low-molecular-weight peptide fractions were detected when strains *L. helveticus* h70 and *L. helveticus* b244 were cultivated in a plant medium with pea protein. Combined media containing milk and plant proteins showed a synergistic effect and contributed to the appearance of new peptide fractions after fermentation with *L. helveticus* h70 and *L. helveticus* b244.

CONCLUSIONS

1. In an initial screening of 50 lactobacillus strains belonging to four species—*Lactobacillus delbrueckii* ssp. *bulgaricus* (30), *Lactobacillus helveticus* (11), *Lacticaseibacillus casei* (4) and *Lactiplantibacillus plantarum* (5)-cultivated on medium with 10% skimmed milk powder, the strains with the highest proteolytic activity selected were *L. helveticus* AB, *L. helveticus* h25, *L. helveticus* h48, *L. helveticus* h70, *L. helveticus* b244 and *Lacticaseibacillus casei* c1. When cultivated on medium with 5% pea protein + 2% glucose and 5% soy protein + 2% glucose, the highest proteolytic activity was observed for *L. helveticus* h70 and *L. helveticus* b244.
2. Pea protein hydrolysate, soy protein hydrolysate and whey protein WP 80 were found to be the strongest activators of the proteolytic system in the selected strains. *L. helveticus* b244 and *L. helveticus* h70 showed the highest specific proteolytic activities in the ranges 16,85–57,29 U/mg protein and 10,61–64,96 U/mg protein, respectively.
3. Strain-specific aminopeptidase activities were demonstrated for four aminopeptidases-leucine-, lysine-, arginine- and proline-aminopeptidases in the selected strains. *L. helveticus* h25 showed the highest specific leucine and lysine aminopeptidase activities (696,76 U/mg protein and 816,20 U/mg protein, respectively). Arginine and proline aminopeptidase activities were detected when *L. helveticus* strains were cultivated in 10% skimmed milk powder and in mMRS with 1% pea protein hydrolysate. *L. helveticus* h48 showed the highest specific arginine and proline aminopeptidase activities in the tested media (4,43 U/mg protein and 3,84 U/mg protein, respectively).
4. *L. helveticus* b244 exhibited the highest ACE-inhibitory activity when cultivated in plant media containing soy and pea protein. ACE-inhibitory activity was also observed for combined milk-plant media (pea and soy) with strains *L. helveticus* h70 and b244. The highest ACE-inhibitory activity was obtained in the plant medium containing 5% soy protein with 2% glucose. *L. helveticus* b244 showed the highest ACE-inhibitory value in all tested media, equal to 75,5 U/mg protein.
5. SDS-Tris-tricine PAGE showed that selected strains *L. helveticus* h70 and b244 actively hydrolyze not only milk proteins but also protein fractions in media containing plant proteins. Electrophoretic analysis confirmed that cultivation of these strains in pea-protein medium yields low-molecular-weight peptides < 6,5 kDa.

6. UHPLC-MS confirmed formation of low-molecular-weight peptide fractions when *L. helveticus* h70 and b244 were cultivated in plant medium with pea protein.
7. Combined media containing milk and plant proteins (pea and soy) had a synergistic effect and contributed to appearance of new peptide fractions after fermentation with *L. helveticus* h70 and b244.

CONTRIBUTIONS

1. Demonstrated that pea protein hydrolysate, soy protein hydrolysate and whey protein WP 80 act as strain-specific activators of proteolytic activity in the selected strains.
2. Demonstrated production of bioactive peptides with ACE-inhibitory activity by selected lactobacilli cultivated in media containing pea and soy protein.
3. For the first time, two new plant-based media containing pea and soy protein were formulated, suitable for cultivation of selected lactobacilli and easily applicable in production processes.
4. For the first time, combined media with milk and plant proteins (pea and soy) were formulated for production of bioactive peptides.

PUBLICATIONS RELATED TO THE DISSERTATION

1. Panayotova, T.M., Urshev, Z.L. and Iliev, I.N., 2023. Specific protease-and aminopeptidase activity of potential bioactive peptide-producing lactobacilli in media with plant protein hydrolysates. BULGARIAN CHEMICAL COMMUNICATIONS, p.57. DOI: 10.34049/bcc.55.C.0006 (Q4)
2. Panayotova, T., Urshev, Z., Angelova, S. and Iliev, I., 2024. Proteolytic activity of different lactic acid bacteria species cultivated on media with plant proteins. Acta Microbiologica Bulgarica, 40(4), pp.520–525. <https://doi.org/10.59393/amb24400412> (Q4)
3. Panayotova, T., Urshev, Z., Yordanova, K. and Iliev, I., 2025. Proteolysis and Peptide Formation from Pea and Soy Protein by Two Lactobacillus helveticus Strains. Acta Microbiologica Bulgarica, 41(2), pp.239–243. <https://doi.org/10.59393/amb25410213> (Q4)

LITERATURE

The full literature review and list of cited references can be found in the dissertation.