Effect of *Lactobacillus acidophilus* Bauer and *Bifidobacterium animalis* ssp. *lactis* BB12 on proteolytic changes in dry-cured loins

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Abstract The effect of the potentially probiotic bacteria strain of *Lactobacillus acidophilus* Bauer and probiotic bacteria *Bifidobacterium animalis* ssp. *lactis* BB12 on proteolytic changes of proteins in dry-cured loins during fermentation and cold storage was studied. Results of the conducted tests demonstrated that the use of probiotic bacteria for the production of dry-cured meats impacts the generation of products of protein proteolysis with high antioxidant activity. The highest antioxidant activity of peptides after fermentation and cold storage was observed in the loin with the strain *B. animalis* ssp. *lactis* BB12 and the loin with the mixture of strains *L. acidophilus* Bauer and *B. animalis* ssp. *lactis* BB12. Qualitative analysis of peptides demonstrated that peptides with weight below 3.5 kDa are characterized by the highest capacity of quenching the ABTS cation radical, including the peptides in loins with the strain *B. animalis* ssp. *lactis* BB12.

Keywords Dry-cured loins · Free amino acids · Peptides · Antioxidant activity

Introduction

Dietary proteins constitute a rich source of bioactive peptides, which are hidden within the native protein in a latent state, requiring enzymatic proteolysis for release. Biologically active peptides are food-derived peptides that exert,

beyond their nutritional value, a physiological, hormone-like effect in the body [1]. Traditional dry-cured loin is a high-quality product with a typical and characteristic texture and flavor. These parameters require a long production process involving salting, post-salting, and ripening steps, which could last up to 12 months. Many biochemical reactions taking place during this process are responsible for its final texture and flavor [2].

During recent decades, there has been a growing consumer demand for probiotic foods, a group of functional foods that are believed to contribute to health. The health benefits associated with probiotic food products are based on the presence of selected viable microorganisms that, when administered in adequate amounts, improve the health of the host [3]. In addition, the sausage matrix offers protection for the survival of the probiotic bacteria through the gastrointestinal tract [4]. Bioactive peptides of muscle tissue proteins of slaughter animals and fish demonstrated an impact on humans that is similar to the impact of other raw product peptides (milk and soya), such as lowering of blood pressure, as well as anti-oxidant, anti-bacterial, and anti-proliferation activity (among others) [5]. The enzymology of dry-fermented loin is quite complex owing to the coexistence of enzymes from both endogenous and microbial origin. Endopeptidases include calpains and cathepsins B, L, H, and D and exopeptidases include microbial enzymes I and II tripeptidase, I, II, III, and IV dipeptidase as well as alanyl, arginyl, methionyl, leucyl, and aminopeptidases [6]. Muscle proteinases appear to be the main enzymes responsible for the initial breakdown of myofibrillar and sarcoplasmic proteins, which are substrates for endogenous cathepsins [7].

This study aims to determine the effect of the potential probiotic strains *L. acidophilus* Bauer and *B. animalis* ssp.

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lactis BB12 on the proteolysis of pork loin during fermentation, dry-curing process, and cooling storage.

Materials and methods

Reagents

All chemicals were of analytical grade and purchased from Sigma–Aldrich, POCH, CHEMPUR® and Spectra/Por®.

Loin preparation

Starter cultures

In this study, one species that was known and fulfilled the criteria required for probiotic strains, *B. animalis* subsp. *lactis* BB12® (Chr. Hansen, Horsholm, Denmark), and one potential probiotic strain *L. acidophilus* Bauer® (collection of the Institute of Fermentation Technology and Microbiology, Faculty of Biotechnology and Food Sciences, Technical University of Łódź, Poland) were used. In the studies performed by Libera et al. [8] and Jaworska et al. [9], it was proved that these two probiotic strains have good technological properties for the production of dry-fermented meat products.

Loin production

Pork loins ($n = 12$) were obtained from the Large White Polish pig breed with the live weight of ~120–130 kg, loins were dry-cured with 28 g of mixture (with the following content: 20 g of marine salt, 9.7 g of curing salt, and 0.3 g of sodium nitrate)/kg of raw meat during 48 h at the temperature of 2 °C. Bacteria strains were transferred onto the meat surface in the amount of 6 log CFU/g of meat and glucose (6 g/kg of meat), after which the loins were left in the fermentation chamber at a temperature of 16 °C and relative air humidity of 70–75% for 30 days. Fermented loins were vacuum-packed and stored at a temperature of 4 °C for 9 months. Qualitative and quantitative tests of proteolysis products were conducted immediately after the fermentation (0) and after 9 months of cold storage (4 °C). The loins were randomly divided in four experimental batches with three loins in each batch:

- C without probiotic starter cultures;
- P1 with probiotic starter cultures *B. animalis* subsp. *lactis* BB12;
- P2 with potential probiotic starter cultures *L. acidophilus* Bauer;
- P3 with mixed two starter cultures (*L. acidophilus* Bauer and *B. animalis* subsp. *lactis* BB12).

At each sampling time, all of the four loins were grounded ($\varnothing$ 3 mm) and used for analyses.

Isolation of peptides

Loin sample with the weight of 1 g was homogenized with 50 ml of distilled water (20 °C) at the speed of 9500 rpm for 2 min. Then, the homogenate was filled up to 100 ml with distilled water and a 5 ml sample was taken. The sample was mixed with 5 ml of 7.5% trichloroacetic acid (TCA) and left for 30 min at a temperature of 4 °C. Further, the sample was filtered through the Whatman® Grade 1 filter paper.

Physico-chemical parameters

pH analysis

The pH of a 10-g sample homogenized with 50 ml of distilled water was measured using a digital pH-meter CPC-501 (Elmetron, Zabrze, Poland) with a combined electrode ERH-111 (Hydromet, Gliwice, Poland). Each sample was analyzed three times. Measurements were performed at 20 °C.

Oxidation–reduction potential (ORP)

The ORP of a 10-g sample homogenized with 50 ml of distilled water was measured using a digital pH/conductivity meter CPC-501 (Elmetron, Zabrze, Poland) with a combined electrode ERPt-13 (Hydromet, Gliwice, Poland). Each sample was analyzed three times. Measurements were performed at 20 °C.

Water activity (a_w)

Water activity measurement was conducted using the LabMaster-aw (Novasina AG, Lachen, Switzerland). Loin sample with the weight of 5 g was placed in the measurement chamber of the apparatus and the measurement was performed at the temperature of 20 °C.

Amino acid analysis

The contents of free amino acids were determined using an automatic amino acid analyzer AAA 400 (Ingos Ltd., Czech Republik) by the method reported by Stadnik and Dolatowski [10]. Free amino acid content was expressed as mg/g of dry matter.

Peptide dialysis

The dialysis of peptides was conducted using tubing membranes manufactured by Spectra/Por®. The

membranes were made from regenerated cellulose with 3.500 MWCO (Daltons)-sized filtered particles. The peptide sample with the volume of 10 ml was introduced into the dialysis tube, and it was placed in 200 ml of PBS saline solution (8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4 ; and 0.24 g K_2HPO_4 dissolved in 100 ml of distilled water). The sample was shaken in a water bath at a temperature of 37 °C for 60 min.

Determination of peptide concentration

The concentration of peptides was determined by the Lowry method [11] using L-leucine as the standard.

Antioxidant activity

The antioxidant activity of peptide extracts was determined according to Re et al. [12] by using a free radical scavenging assay with 2,2'-azobis(3-ethylbenzotiazolino-6-sulfonate) (ABTS^{+}) as the source of the free radicals. For the ABTS assay, 0.2 ml of peptide extract was mixed with a 2 ml solution of ABTS^{+} . Absorbance at 734 nm was measured using a UV-Vis spectrophotometer (Nicole Evolution 300, Thermo Elektron Corporation) immediately and after 6 min of incubation. The antiradical activity was related to Trolox (an analogue of vitamin E) and expressed as mg of Trolox per mg peptides. All assays were performed in triplicates.

Electrophoresis

The process of electrophoretic separation of peptides was conducted in the Mini-PROTEAN® Tetra Cell apparatus according to the Laemmli method [13] using 14% separating gel and 5% stacking gel. First, 0.005 ml of mercaptoethanol and 0.045 of Laemmli buffer were added to 0.15 ml of peptide mixture, after which they were heated for 5 min at a temperature of 95 °C. Thus, the prepared sample was transferred onto gel. Subsequently, electrophoresis was conducted at a constant voltage of 40 V for ~6 h. After electrophoresis, the gel was placed in the staining buffer (Coomassie Brilliant Blue R-250 Staining Solution manufactured by Bio-Rad). After 1 h of staining, the gel was placed in the destaining buffer (Coomassie Brilliant Blue R-250 Destaining Solution manufactured by Bio-Rad). Next, the gel was placed in the BIO-RAD Gel Doc™ EZ Imager, and it was documented in the form of photograms.

Statistical analysis

Statistical characteristics of the sample were prepared (mean value, standard deviation, and standard error) as

well as ANOVA variance analysis (Excel 2013; Microsoft Corp., Redmond, WA, USA) was conducted; the significance of differences between the samples was defined by means of Tukey test at the confidence level of 0.05. All measurements were performed in triplicate, and the data were expressed as mean $\pm$ SE.

Results and discussion

Changes in the physico-chemical parameters

The analysis of the acidity of loins demonstrated a lack of statistically important differences of pH values between the samples during cold storage (Table 1). After 9 months of storage, the control sample and the sample with the strain *L. acidophilus* Bauer were characterized by the lowest pH values, whereas the sample with the mixture of strains *B. animalis* ssp. *lactis* BB12 and *L. acidophilus* Bauer as well as the sample with the strain *B. animalis* ssp. *lactis* BB12 were characterized by the highest pH values. The product acidity provides appropriate conditions for the activity of proteolytic enzymes [7, 14]. On the basis of the tests performed by different authors, it was established that the products with lower pH values were characterized by higher protein degradation [14, 15]. This suggests that proteolysis in the products with higher acidity is visible at the beginning of the dry-curing process, i.e., directly after fermentation, and it is the result of the rapid increase of acidity [16, 17].

Results of the ORP measurements (Table 1) demonstrated that both the application of bacteria strains and storage time significantly influenced the ORP values of dry-cured loins. The highest values of ORP were observed directly after fermentation in the control sample and this value was higher by approximately 40–70 mV from those of probiotic loins. After 9 months of storage, the increase of ORP was determined in all tested samples. The highest ORP directly after fermentation and after 9 months of cold storage in the control sample indicates the increase of oxidation. The lower values of ORP in probiotic loins both directly after fermentation and after 9 months of storage indicate elevated oxidation suppression. It could be inferred that in the control loin, the microflora of meat has an impact on the direction of changes during fermentation and dry-curing processes. The obtained values of ORP and their diversification may indicate the participation of low molecular peptides with oxidation-reducing properties in the suppression of oxidation–reduction processes of the entire tissue as a result of probiotic bacteria growth and the inactivation of some bacteria in the meat environment.

After 9 months of storage, significant ($\alpha \leq 0.05$) reduction of water activity was observed. The water

Table 1 Physico-chemical parameters of loins (n = 12)

Sample	Storage time (months)	Parameters		
		pH	ORP [mV]	Water activity (a_w)
C	0	5.65 ± 0.01 ^{aA}	377.27 ± 0.34 ^{aA}	0.955 ± 0.0004 ^{aA}
	9	6.22 ± 0.02 ^{bA}	410.30 ± 0.63 ^{bA}	0.909 ± 0.0017 ^{bA}
P1	0	5.46 ± 0.01 ^{aB}	332.63 ± 0.47 ^{aBC}	0.956 ± 0.0002 ^{aA}
	9	6.36 ± 0.01 ^{bA}	362.67 ± 0.44 ^{bBD}	0.922 ± 0.0023 ^{bA}
P2	0	5.65 ± 0.01 ^{aA}	307.10 ± 0.47 ^{aCD}	0.954 ± 0.0003 ^{aA}
	9	6.27 ± 0.01 ^{bA}	382.70 ± 1.27 ^{bBC}	0.920 ± 0.0010 ^{bA}
P3	0	5.55 ± 0.01 ^{aAB}	330.57 ± 1.42 ^{aC}	0.953 ± 0.0006 ^{aA}
	9	6.37 ± 0.01 ^{bA}	372.70 ± 0.26 ^{bCD}	0.917 ± 0.0003 ^{bA}

^{a-b} Within the same treatment, means followed by the common letter do not differ significantly ($P < 0.05$)

^{A-D} Within the same storage time, means followed by the common letter do not differ significantly ($P < 0.05$)

activity in samples after fermentation and after 9 months of storage was similar, and it could not be the factor of increased growth of any species or strain of bacteria. Pomponio et al. [18] observed a similar water reduction in the research discussed herein in relation to all samples. The water reduction leads to the increase in the salt content, which blocks the activity of aminopeptidases, resulting in decrease in the amount of peptides. Such tendency could be observed in this study after 9 months of storage, particularly in the control sample and in the sample with the mixture of strains *L. acidophilus* Bauer and *B. animalis* ssp. *lactis* BB12.

Free amino acid content after fermentation and storage

The analysis of free amino acid profile in loins directly after fermentation indicates that the sample with the strain *L. acidophilus* Bauer is characterized by the highest content of alanine, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tyrosine, and valine (Table 2). The analysis of free amino acids directly after the fermentation of loins with the probiotic strain *B. animalis* ssp. *lactis* BB12 and the addition of strains *L. acidophilus* Bauer and *B. animalis* ssp. *lactis* BB12 demonstrated the highest content of glutamic acid (P1 = 0.75 mg/g and P3 = 0.76 mg/g). The highest content of arginine directly after the fermentation process in the P1 and P2 samples with the addition of a single species of probiotic starter culture (0.50 and 0.52 mg/g, respectively) is quite significant. This value was almost two times higher than that in the control sample (0.26 mg/g). The increase of free amino acids was observed after 9 months of storage in all samples, out of which the highest values were observed in the control sample and in the sample inoculated with *B. animalis* ssp. *lactis* BB12 (Table 2). The highest values of glycine, isoleucine, leucine, lysine, methionine, ornithine,

phenylalanine, and threonine were observed in the control sample. The P3 sample with the strain mixture contained eight free acids (glycine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine) with the lowest concentration after 9 months of storage. During the analysis of the obtained free amino acid profile of dry-cured loins after 9 months of cold storage in anaerobic conditions, much higher concentrations of glutamic acid could be observed only in the samples inoculated with probiotic bacteria when compared with the control sample without the addition of the starter culture. When summarizing the obtained results, it could be stated that the highest growth of all free amino acids during 6 months of storage was observed in the P1 sample and it totaled 9.96 mg/g, whereas the lowest growth of 4.92 mg/g was observed in the P3 sample.

After 9 months of storage, the arginine content was the highest in probiotic loins and it totaled 0.25 mg/g, which was 10 times higher than that in the case of the control sample (0.02 mg/g). Probiotic strains change the quantitative and qualitative level of amino acids in the samples [10]. The change factor can be the suppression of natural activity of environmental micro-culture that exists in the raw meat [19]. The types of *Bifidobacterium* and *Lactobacillus* that were used in the discussed conditions of technological process require certain amino acids for their growth, such as arginine, isoleucine, leucine, tyrosine, cysteine, and valine [20, 21]. In the obtained test results, amino acids are present in the product as free amino acids or in peptides with low molecular weight. Gomes et al. [22] suggested that *L. acidophilus* Ki hydrolyze milk proteins into low molecular peptides, which can be used to promote bacterial growth and acidify the environment, which in turn leads to the growth of other bacteria species or strains, such as *B. lactis* Bo. Interactions similar to the research discussed herein have been observed in the presence of other bacteria strains, such as *Bifidobacterium* and *Lactobacillus*,

Table 2 The content (expressed as mg/g of dry matter) of free amino acids after fermentation and 9 months of cold storage

Amino acids	C		P1		P2		P3	
	0	9	0	9	0	9	0	9
Ala	0.99 ^{aB}	0.87 ^{bC}	0.72 ^{aC}	1.35 ^{bAB}	1.09 ^{aA}	1.45 ^{bA}	0.90 ^{aB}	1.29 ^{bB}
Arg	0.26 ^{aB}	0.02 ^{bB}	0.50 ^{aA}	0.27 ^{bA}	0.52 ^{aA}	0.28 ^{bA}	0.30 ^{aB}	0.21 ^{aA}
Gly	0.61 ^{aA}	1.79 ^{bA}	0.46 ^{aB}	0.95 ^{bB}	0.56 ^{aAB}	0.95 ^{bB}	0.47 ^{aB}	0.73 ^{bC}
His	0.54 ^{aB}	0.75 ^{bC}	0.32 ^{aC}	1.14 ^{bB}	0.69 ^{aA}	1.21 ^{bA}	0.58 ^{aB}	1.15 ^{bB}
Ile	0.51 ^{aA}	1.01 ^{bA}	0.39 ^{aB}	0.96 ^{bAB}	0.56 ^{aA}	0.85 ^{bB}	0.50 ^{aA}	0.72 ^{bC}
Glu	0.51 ^{aB}	0.08 ^{bD}	0.75 ^{aA}	2.78 ^{bA}	0.49 ^{aB}	2.41 ^{bB}	0.76 ^{aA}	1.79 ^{bC}
Leu	0.95 ^{aA}	1.79 ^{bA}	0.74 ^{aB}	1.51 ^{bB}	0.98 ^{aA}	1.42 ^{bB}	0.80 ^{aB}	1.23 ^{bC}
Lys	1.54 ^{aB}	3.26 ^{bA}	1.14 ^{aD}	2.11 ^{bC}	1.72 ^{aA}	2.47 ^{bB}	1.35 ^{aC}	1.93 ^{bD}
Met	0.38 ^{aA}	0.65 ^{bA}	0.30 ^{aA}	0.57 ^{bA}	0.37 ^{aA}	0.57 ^{bA}	0.29 ^{aA}	0.46 ^{bA}
Phe	0.57 ^{aA}	0.99 ^{bA}	0.44 ^{aB}	0.87 ^{bB}	0.57 ^{aA}	0.95 ^{bAB}	0.45 ^{aB}	0.77 ^{bC}
Pro	0.30 ^{aB}	0.45 ^{bB}	0.22 ^{aB}	0.67 ^{bA}	0.29 ^{aB}	0.69 ^{bA}	0.52 ^{aA}	0.54 ^{aB}
Ser	0.23 ^{aC}	0.70 ^{bC}	0.37 ^{aB}	1.37 ^{bA}	0.48 ^{aB}	1.36 ^{bA}	0.76 ^{aA}	1.07 ^{bB}
Thr	0.36 ^{aA}	1.57 ^{bA}	0.35 ^{aA}	0.67 ^{bB}	0.27 ^{aA}	0.53 ^{bC}	0.30 ^{aA}	0.47 ^{bC}
Tyr	0.35 ^{aAB}	0.37 ^{aC}	0.29 ^{aB}	0.53 ^{bAB}	0.45 ^{aA}	0.61 ^{bA}	0.39 ^{aA}	0.45 ^{aBC}
Val	0.68 ^{aAB}	1.61 ^{bA}	0.47 ^{aC}	1.67 ^{bA}	0.78 ^{aA}	1.26 ^{bB}	0.63 ^{aB}	1.11 ^{bC}
Cys	nd	nd	nd	nd	nd	nd	nd	nd
Total	8.78 ^{aA}	15.91 ^{bB}	7.46 ^{aB}	17.42 ^{bA}	9.82 ^{aA}	17.01 ^{bA}	9.00 ^{aA}	13.92 ^{bC}
Urea*	2.92 ^{aA}	8.30 ^{bA}	2.17 ^{aA}	1.57 ^{bD}	2.38 ^{aA}	2.78 ^{bC}	nd ^{aB}	3.90 ^{bB}

Nd not detected; 0: after fermentation; 9: after 9 months of cold storage

^{a-b} Within the same treatment, means followed by the common letter do not differ significantly ($P < 0.05$)

^{A-D} Within the same storage time, means followed by the common letter do not differ significantly ($P < 0.05$)

* Amino acids degradation product

by Gomes et al. [22] and Altier et al. [23]. This phenomenon was confirmed by Aro et al. [24], who did not observe significant differences in the product acidity between the samples after 21 days of fermentation of sausages with different bacteria strains (*Lactobacillus sakei* D-1001, *Staphylococcus carnosus* SB-61, *Staphylococcus xylosus*, and *Pediococcus pentosaceus*); however, they observed significant growth in the content of free amino acids in these samples. The content of free amino acids in the product impacts the rate of their degradation into biogenic amines by appropriate enzymes, the activity of which is, on the other hand, inhibited by the content of kitchen salt and low water activity [25]. Similar dependencies were observed by Flores et al. [26] and Schivazappaa et al. [27]. These dependencies are confirmed by urea values (Table 2). The content of urea, which is the degradation product of amino acid, directly after fermentation was highest in the control sample (2.92 mg/g). Similarly, after 6 months of storage, it was the highest in the control sample (8.30 mg/g), which was statistically significant. It could be stated that greater dissociation of amino acids could be observed in the control sample than in the samples with probiotic bacteria. This could also be observed in the reduced sensory quality of these products [28].

The obtained results indicate the purposefulness of probiotic bacteria application for the production of dry-cured meat products owing to their influence on meat proteins degradation. Similar dependencies were observed by Casaburi et al. [14], who noted the increase of glutamic acid and alanine in relation to the changes of free amino acid content in sausages with the strains *S. xylosus* and *Lactobacillus curvatus*. Fadda et al. [20] stated in their research that LAB bacteria promote, to various degrees, the release of certain amino acids. They demonstrated that *L. curvatus* impacts the generation of high quantities of glutamic acid, alanine, histidine, arginine, and lysine, whereas *L. sakei* impact the level of glutamic acid, alanine, gamma aminobutyric acid, leucine, phenylalanine, and ornithine. The research by other authors also confirmed similar changes of the quantity of free amino acids in the content of traditionally fermented sausages to the research discussed herein, caused by starter cultures with the specific content of bacteria [19].

Peptide concentration in dry-fermented pork loins

The highest concentrations of peptides directly after fermentation (Fig. 1) were observed in the sample with the

strain *L. acidophilus* Bauer and in the control sample. The sample with the strain *B. animalis* ssp. *lactis* BB12 directly after fermentation had the lowest content of peptides. The highest peptide content after 9 months of storage was noted for the sample with the strain *L. acidophilus* Bauer, whereas the lowest content was observed in the sample with the mixture of strains *L. acidophilus* Bauer and *B. animalis* ssp. *lactis* BB12 and in the control sample.

The quantitative analysis of peptides indicates that the highest peptide content directly after fermentation and after 9 months of storage was observed in the sample with the potentially probiotic strain *L. acidophilus* Bauer. After 9 months of storage, the highest increase of peptide content in comparison with the value directly after fermentation was observed in the P2 sample, whereas the least increase was observed in the control sample. According to some studies, certain strains, i.e., *L. curvatus* and *L. sakei* impact the hydrolysis of sarcoplasmatic proteins into peptides and the subsequent hydrolysis of peptides into amino acids [21].

The conducted dialysis of peptides of dry-cured loins after 9 months of storage (Fig. 2) using membranes with permeability of 3500 Da demonstrated significant quantities of peptide fractions with a weight <3.5 kDa in specific samples. The highest content of low molecule peptide fraction was observed in the sample with the strain *B. animalis* ssp. *lactis* BB12, and the lowest one (almost twice) was observed in the sample with the strain *L. acidophilus* Bauer. The highest peptide content with a weight >3.5 kDa was observed in the sample with the strain *L. acidophilus* Bauer and *B. animalis* ssp. *lactis* BB12, whereas the lowest content was observed in the sample with the strain *B. animalis* ssp. *lactis* BB12.

The characteristics of peptides with a weight below 3.5 kDa in loins after 9 months of storage indicated their lowest content in the sample with the strain *L. acidophilus*

Fig. 1 Peptides content in dry-fermented loins during cold storage ^{a-b}Within the same treatment, means followed by the *common letter* do not differ significantly ($P < 0.05$) ^{A-C}Within the same storage time, means followed by the *common letter* do not differ significantly ($P < 0.05$)

Fig. 2 Content of peptides with weight below and above 3.5 kDa in loins after 9 months of storage ^{a-b}Within the same treatment, means followed by the *common letter* do not differ significantly ($P < 0.05$) ^{A-C}Within the same molecular weight, means followed by the *common letter* do not differ significantly ($P < 0.05$)

Bauer and their highest content in the sample with the strain *B. animalis* ssp. *lactis* BB12. Quantitative and qualitative analyses of peptide fractions above and below 3.5 kDa indicate significant changes in the activity of hydrolytic enzymes. A greater share of fractions with a low molecule weight indicates the increased activity of the peptidases of probiotic bacteria, which was also confirmed by the electrophoretic separation of peptides.

Electrophoretic separation of peptide fractions with weight >3.5 kDa (Fig. 3) on polyacrylamide gel demonstrated the quantitative and qualitative differentiation of peptides in the samples.

The lowest quantity of low molecule peptides was observed in the control sample (17.9 and 10.2 kDa), whereas the highest one was observed in the sample with the mixture of strains *L. acidophilus* Bauer and *B. animalis* ssp. *lactis* BB12 (18.4, 16.6, 6.9, and 6.5 kDa) and in the sample with the strain *L. acidophilus* Bauer (18.2, 16.6, 10.9, and 7.1 kDa). The fraction of peptides with a weight of approximately 18 kDa is present in all samples. The analysis of electrophoretic separation on polyacrylamide gel also showed a fraction with a weight of approximately 16 kDa in probiotic loins (P1, P2, and P3). The fractions with low molecule weight were discovered in the C sample

Fig. 3 Photographs of electrophoretic separation of peptides from loins after 9 months of storage. Std: standard weight in kDa: (1) Myoglobin 16.9; (2) Alfa-Lactalbumin 14.4; (3) Aprotinin 6.5

(approximately 8 kDa), in the sample with the strain *B. animalis* ssp. *lactis* BB12 (approximately 9 kDa), in the sample with the strain *L. acidophilus* Bauer (approximately 10 and 7 kDa), and in the samples with the mixture of strains *L. acidophilus* Bauer and *B. animalis* ssp. *lactis* BB12 (approximately 7 and 6 kDa). A greater share of fractions with a low molecule weight indicates the increased activity of peptidases of probiotic bacteria, which hydrolyze polypeptides into smaller molecules and even into amino acids. An important factor of the biological properties of peptides is their direct use by the organism, which is possible owing to the absorption of low molecule fractions of peptides into the bloodstream [29].

The increase of fraction content with weight <13.7 kDa was observed in the research conducted by Toldrá and Flores [2] and Toldrá et al. [30]. This peptide fraction in the discussed research was visible in all the samples just after fermentation, and their greatest quantities were observed after 9 months of cold storage. The presence of these qualities is mainly related to the degradation of myofibrillar proteins, the breakdown of which was always observed at the end of the meat maturing process, which was reported in the research by Rodríguez-Núñez et al. [31] and Ruiz-García et al. [32].

Antioxidant activity of peptides of dry-cured loins

The analysis of antioxidant properties of peptides of dry-cured loins after fermentation and cold storage (Fig. 4) indicates that the highest antioxidant activity directly after fermentation was observed in the sample with the strain *B. animalis* ssp. *lactis* BB12 and the sample with the mixture of strains *L. acidophilus* Bauer and *B. animalis* ssp. *lactis* BB12 (1.84 and 1.78 mg of Trolox/mg of peptides, respectively).

Fig. 4 Antioxidant properties of peptides in dry-fermented loins during cold storage ^{a–b}Within the same treatment, means followed by the common letter do not differ significantly ($P < 0.05$) ^{A–B}Within the same storage time, means followed by the common letter do not differ significantly ($P < 0.05$)

The lowest antioxidant activity was observed in the sample with the strain *L. acidophilus* Bauer and in the control sample. The increase of activity inhibiting the ABTS cation radical was observed after 9 months of cold storage in all samples. The highest antioxidant activity was observed in the sample with the mixture of strains *L. acidophilus* Bauer and *B. animalis* ssp. *lactis* BB12. Statistically significant ($\alpha \leq 0.05$) increase of the antioxidant activity of peptides was observed in the control sample between the value obtained after fermentation and that obtained after 9 months of storage in the P2 sample.

A statistically significant differences in antioxidant activity of peptides directly after fermentation was observed between the control sample and the P1 sample (by 0.88 mg of Trolox/mg of peptides), samples P1 and P2, and samples P2 and P3. It could be inferred that the strain *B. animalis* ssp. *lactis* BB12 had a significant share in the quantitative and qualitative peptide differentiation. Similar antioxidant values of the tested loins were demonstrated after 9 months of storage. The highest antioxidant activity after 9 months of storage was observed in the sample with the mixture of strains *L. acidophilus* Bauer and *B. animalis* ssp. *lactis* BB12. A research on dry-cured hams conducted by Escudero et al. [33, 34] specified a few peptides with antioxidant activity. The highest activity was demonstrated by peptides such as SAGNPN, AAAAG, IHSGSVG, NVLVG, NAAKL, and GLAGA. Vaštag et al. [35] demonstrated high antioxidant activity of water extracts obtained from the proteins of fermented Serbian Petrov sausage (*Petrovská Kolbasa*) during the in vitro maturing process.

Results of antioxidant properties of peptides with different molecular weights indicated that peptides with low molecular weight are characterized by higher antioxidant activity than the peptides with weight above 3.5 kDa (Fig. 5).

The highest antioxidant activity of low molecule peptides was observed in the sample with the strain *B. animalis* ssp. *lactis* BB12, and the lowest one was observed in the sample with the strain *L. acidophilus* Bauer. The antioxidant activity of the peptides of the P1 sample was higher than that of the P2 sample ($\alpha \leq 0.05$), which was statistically significant. The peptides in loins with the strain *B. animalis* ssp. *lactis* BB12 and in loins with the mixture of *B. animalis* ssp. *lactis* BB12 and *L. acidophilus* Bauer were characterized by statistically significant greater activity in comparison with the peptides from the control and P2 samples. The peptides with the weight exceeding 3.5 kDa showed similar antioxidant activity in all tested samples, approximately 0.06 mg of Trolox/mg of peptides; however, their quantity was different in specific samples.

Results of the research demonstrated that the use of probiotic bacteria for the production of dry-cured meats impacts the generation of products of protein proteolysis

Fig. 5 Antioxidant properties of peptides with different molecular weight in loins after 9 months of storage ^{a-b}Within the same treatment, means followed by the *common letter* do not differ significantly ($P < 0.05$) ^{A-B}Within the same molecular weight, means followed by the *common letter* do not differ significantly ($P < 0.05$)

with high antioxidant activity. The highest antioxidant activity of peptides after fermentation and cold storage was observed in the loin with the strain *B. animalis* ssp. *lactis* BB12 and loin with the mixture of strains *L. acidophilus* Bauer and *B. animalis* ssp. *lactis* BB12. Qualitative analysis of peptides demonstrated that peptides with the weight below 3.5 kDa are characterized by the highest capacity of quenching the ABTS cation radical.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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