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INFLUENCE OF *BACILLUS SUBTILIS* FERMENTATION ON CONTENT OF SELECTED MACRONUTRIENTS IN SEEDS AND BEANS

– Research paper –

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Abstract: In this study, five plant matrices (pea, mung bean, lentils, soy and sunflower) were fermented using *Bacillus subtilis* var. natto. Then the process influence on the content of fatty acids and proteins was evaluated, depending on the fermentation length. Fermentation was conducted for 144 hours in controlled conditions of temperature and relative humidity (37°C, 75%). Samples for tests were collected every 24 hours. Gas chromatography coupled with triple quadrupole tandem mass spectrometry (GC-MS/MS) was used to evaluate fatty acids content in fermented seeds. Their composition was expressed as a percentage of the total quantity of fatty acids. The protein content in plant matrices was analysed with the modified Bradford protein assay, using the TECAN apparatus with the i-Control software, of the wave length of ʎ=595 nm. Studies showed that the prolonged fermentation time influenced an increase of polyunsaturated fatty acids (PUFA) content in all studied seeds. Promising results were obtained for soy, sunflower, and lentil seeds, amounting to 3.6%; 68.7% and 67.7%, respectively. This proves that the process of seed fermentation can be effectively used to increase their nutritional value.

Key words: *Bacillus subtilis* (natto), bacterial fermentation, seeds/beans, fatty acids, proteins

INTRODUCTION

Fermentation is a metabolic process in which organic compounds, mainly glucose, are oxidised. Microorganisms and their enzymes play a crucial role in fermentation (Dimidi et al., 2019). Fermentation is used to improve sensory parameters, increase the nutritional value, and extend the shelf life of food products. Fermented food is considered to be one of functional food groups. It was demonstrated that fermented products are rich in bioactive compounds able to reduce the inflammatory activity (Selhub et al., 2014), and influence brain functions, as well as having anti-oxidative effects (Marco et al., 2017). This food is also a good source of organic acids and B vitamins, due to presence and activity of fermentation bacteria (Ligenza et al., 2021). Furthermore, the fermentation process enriches a product with high quality proteins and reduces antinutrients content (Khattab & Arntfield, 2009).

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Pulses are a source of fatty acids and proteins, and additionally contain other valuable nutrients, like fibre or vitamins, and their content may change following treatment or processing, e.g. fermentation (Nowak & Żmudzińska-Żurek, 2007). Microorganisms used for fermentation have a potentially synergistic effect, helping to improve quality of fermented products. *Bacillus* bacteria belong to probiotic bacteria responsible for reduction of hard to digest oligo- and polysaccharides. Fermentation can improve digestibility of pulse proteins by minimising levels of antinutrients, i.e., phenol compounds and tannins, and inhibiting digestive enzymes (e.g. trypsin and chymotrypsin inhibitors) (Melini et al., 2019). Additionally, fermentation with *Bacillus subtilis* (B. *subtilis*) may be used as an effective strategy to strengthen proteolysis and the anti-oxidative effect, while the fermented seeds can become functional food (Li & Wang et al., 2021).

Previous studies on fermentation of 10 different plant substrates, including pulses and sunflower, focused on natural forms of vitamin K2 MK-7

(Słowik-Borowiec et al., 2021). Following conducted experiments, clear data was obtained indicating that the sunflower, the mung bean, the pea and the lentils, are a very good source of that vitamin and an alternative to soy. Thus, at successive steps, it was decided to continue more extensive studies on selected plant matrices, focusing on the content of other bioactive ingredients.

Fats belong to main nutrients and are a building material and a source of energy. They supply the body with fat soluble vitamins and are a source of fatty acids, which composition influences their nutritional value. Fatty acids differ in their chemical structure. Saturated fatty acids (SAFA) are a source of energy. Their excessive consumption negatively influences the lipid profile of human blood. They may increase LDL cholesterol and triglyceride (TG) levels, which contribute to a higher risk of cardiovascular diseases (Hunter et al., 2010). Monounsaturated fatty acids (MUFA) are synthesised in the human body and reduce cholesterol blood levels. Their recommended daily consumption corresponds to 20% of energy demand (Panel on Macronutrients, 2005). Polyunsaturated fatty acids (PUFA) are not synthesised in the human body, and they play various functions in it. N-3 and n-6 fatty acids, also called essential unsaturated fatty acids (EFA), have the highest biological value. They contribute to prevention and alleviation of numerous diseases, like diabetes, obesity, or hypertension. N-3 fatty acids have the health-improving anti-atherosclerotic effect by reducing cholesterol plasma levels. These acids also have anticoagulation effect, reducing platelet adhesion. Furthermore, they contribute to normalisation of blood pressure and have anti-inflammatory and anti-allergic effect (Rudzińska & Przybylski, 2019). DHA and EPA are particularly important, and they play a role in processes of remembering and improving concentration, and reduce the risk of development of dementia in elderly people (Swanson et al., 2012). Fatty acids have a diversified structure, and they are analysed on a basis of the length of their hydrocarbon chain, saturation level, or presence of functional groups. Gas chromatography with mass spectrometry is one of the most popular techniques for determining fatty acids. As these compounds are characterised by low volatility, the first step aims at achieving their derivatives in from of methyl esters. Then transesterifications are performed, followed by qualitative and quantitative chromatographic analyses (Selected methods of food analysis, 2003).

Proteins belong to essential ingredients of food and sources of energy, beside carbohydrates and fats (Ciborowska & Rudnicka, 2019). They are a building material of every living organism, being a main component of muscles and tissues. They participate in DNA replication and catalyse chemical reactions. They also contribute to transport of molecules and form a part of immune bodies. They contribute to blood pressure regulation and form a structural support of cells (Rehman et al., 2021). Plant products are an excellent source of protein, because they supply all essential amino acids and many other nutrients, including fibre. Popular sources of these ingredients are cereal products, pulses, and nuts (Hoffman & Falvo, 2004). Protein levels can be evaluated with many methods based on their specific properties, like interactions with other proteins, fats and sugars (Food analysis, 2016). Proteins are determined using indirect and direct methods. The majority of them is based on spectrophotometry employing characteristic colour reactions of proteins and peptides. These methods differ in their sensitivity, detection ranges, and presence of additional substances. One of them is the Bradford protein assay, in which the dye Coomasie Blue binds with protein molecules, and changes the colour from brown red to blue (Selected methods of food analysis, 2003).

The aim of this study was to conduct the fermentation process in 5 plant matrices using *B. subtilis* var. natto in controlled conditions of temperature and relative humidity, and then to determine fatty acids and protein content depending on the fermentation length. Fermentation was monitored by determining levels of those macronutrients.

MATERIALS AND METHODS

Fermentation

Fermentation of plant products was conducted on a basis of the paper by Słowik-Borowiec et al. (2021). The reference strain *B. subtilis 3349* ATCC-15245 from American Type Culture Collection (USA) was used in the experiments. Bacteria (from a single colony) were inoculated into the culture medium LB MIX (BTL sp. z o. o., Poland) (1% v/v) containing peptone K (10.0 g/L), sodium chloride (10.0 g/L), yeast extract (5.0 g/L), and distilled water. Then the cultures were incubated in a laboratory oven at 37°C for 24 hours, to obtain a suspension of microorganisms of density of ~1.0 (OD=600 nm). The obtained *B. subtilis* seed stock was added (10%

v/v) to a production media containing: 3% of soy protein isolate (Exeller® Sp. z o.o, Poland); 0.75% of sucrose (Chempur, Poland); 0.75% of malt extract (BTL sp. z o. o., Poland) and 1.5% of yeast extract (BTL sp. z o. o., Poland). This way, a concentration of 1 x 10^8 cells/mL was obtained.
The study material consisted of seeds/beans of pea, mung bean, lentils, soy and sunflower obtained from organic crops (a sample of 50 g of dry matter of each product), which were later washed, rinsed and soaked multiple times, and then sterilised in an autoclave (Classic 2100, Prestige Medical, UK) at 126°C for 1.5 hours. The fermentation process was conducted on a solid medium. 10 mL of the production medium and 10 mL of water were added to glass beakers containing sterile beans/seeds. The entire contents of beakers were mixed and closed with a cellulose stopper. Samples for initial determination of fatty acids and protein were collected from the material prepared as described above. The fermentation process was conducted in a chamber for thermal studies (KBC–100, Wamed, Polska) at a temperature of 37°C and relative humidity of 75% for 144 hours. Samples for tests were collected after each 24 hours of fermentation. The entire experiment was conducted in sterile conditions. When the fermentation ended, the seeds/beans were stored at 4°C for 7 days.

Fatty acids determination
To determine fatty acid levels in the fermented plant material, the method described in earlier publications (Potocki et al. 2020; Rogóż et al. 2021; Szpyrka et al. 2020;) was used. The analytical range of the method covered determination of 37 fatty acids.

Sample extract preparation
Samples of fermented seeds/beans were dried (S–40, Alpina, Poland) to dry weight at 60°C for 6 hours. The dried samples were ground in a mortar. 10 mg of fermented seeds, dry weight, were weighed into a 2 mL chromatographic vial, and then 25 µL of an internal standard C15:0 (1000 µg/mL, 99%, Sigma Aldrich, Poland), 200 µL of dichloromethane:methanol mixture (2:1, v/v) (Honeywell Speciality Chemicals Seelze GmbH, Germany), and 300 µL 0.6 M of HCl solution in methanol (35-38%, p.a purity, Chempur, Piekary Śląskie, Poland) were added. The vials were stopped and their contents were shaken for 1 minute. At the further stage, the vials with their contents were placed in a dryer heated to 85°C±3°C for 1 hour. After that time, the samples were removed from the dryer and cooled to the room temperature. After cooling, 1 mL of petroleum ether (the fraction boiling at 40-60°C, Chempur, Piekary Śląskie, Poland) was added and the samples were shaken again for 1 minute. Then the vials were left for 1 hour to separate phases. 100 µL were collected from the top phase (supernatant), transferred to a 2 mL chromatographic vial, and 400 µL of petroleum ether were added.

Preparing standard solutions of fatty acids
Linearity was established on a basis of 5-point calibration curves. Lipids calibration standards were prepared by diluting the standard solution (37 Component FAME Mix, Supelco, USA) with petroleum ether, obtaining concentrations of 1-6; 0.5-3; 0.25-1.5; 0.05-0.3; and 0.025-0.15 µg/mL.

Chromatographic analysis
The methyl esters content was determined using a gas chromatograph (GC-MS/MS QQQ), model 7890A, Agilent Technologies, Palo Alto, CA, USA, coupled with a mass detector, model 7000) in the single ion monitoring (SIM) mode. The source temperature was 230°C. The electron ionisation (EI), the temperature programme of 40-260°C and the column HP-5 MS (Ultra Inert/30 m × 0.25 mm I.D. × 0.25-µm) were used. Mass Hunter Software (version B.07.06, Agilent Technologies, Palo Alto, CA, USA, 2010) was used for instrument control, and data acquisition and processing. The total analysis time was 28 minutes.

Validation parameters
The method for determination of fatty acid methyl esters (FAME) was validated, and the obtained parameters were satisfactory in terms of linearity, the limit of quantification (LOQ) and the precision. The LOQ for each fatty acid was calculated as a signal to noise ratio (S/N) of 10: 1, and ranged from 0.025-0.15 mg/kg for individual fatty acids. The linearity of each analyte was determined from 5-point calibration curves and ranged from 0.972-1. Recovery studies were performed for samples (n=5) with the addition of an internal triglyceride standard (ISTD, tripentadecanoin), achieving the recovery of 74%, while the precision (RSD%) was expressed as relative standard deviations, and amounted to 6%.

Protein content determination
Plant extract preparation
0.5 g of fermented seeds were weighed (Scale 220/X, Radwag, Poland) into 2 mL Eppendorf-type tubes. Then 0.5 mL of phosphate buffer, ca. 0.5 mL of sterile glass spheres, and 0.5 mL of 2% SDS (sodium dodecyl sulphate) (Sin et al., 2017; Mohammad Saleh et al. 2010) were added to each sample. The tubes were stopped and their contents

were shaken on a vortex (Vortex BenchMixerTM, Benchmark, Edison, NY, USA) at the highest rotations for about 2 minutes. Then the extracts were centrifuged (5804R, Eppendorf, Hamburg, Germany) at 15000 rpm in 4°C for 15 minutes. The supernatant was used at further stages of protein analyses.

Preparing of reagents and standard solutions

Phosphate buffer: NaH_2PO_4 solution (i) (1.39 g dissolved in H_2O in a 50 mL measuring beaker); Na_2HPO_4 solution (ii) (5.36 g dissolved in H_2O in a 100 mL measuring beaker). The buffer was prepared by mixing 16 mL of solution (i) and 84 mL of solution (ii), and filling to 200 mL with water (Saleh et al., 2010).

Bradford reagent: 100 mg of Coomassie Brilliant Blue G-250 were dissolved in 50 mL of 95% ethanol, then 100 mL of 85% of phosphoric acid were added; then on the second day, when the dye dissolved completely, the distilled water was added to obtain 1 L of the solution (Bradford, 1976; Saleh et al., 2010).

To determine a 5-point standard curve, solutions of bovine serum albumin (BSA) were prepared as a standard, at concentrations ranging from 0.125 to 1.000 mg/mL.

Spectrophotometric determination

The supernatant and the Bradford reagent were added to each well of a white 96-well plate. The determinations were performed in triplicates. The absorbance was measured using the TECAN apparatus (Infinite ®, model M200) with the i-Control software, at the wave length of ʎ= 595 nm versus a control reagent sample. Proteins concentrations in the studied samples were calculated from the standard curve (Figure 1).

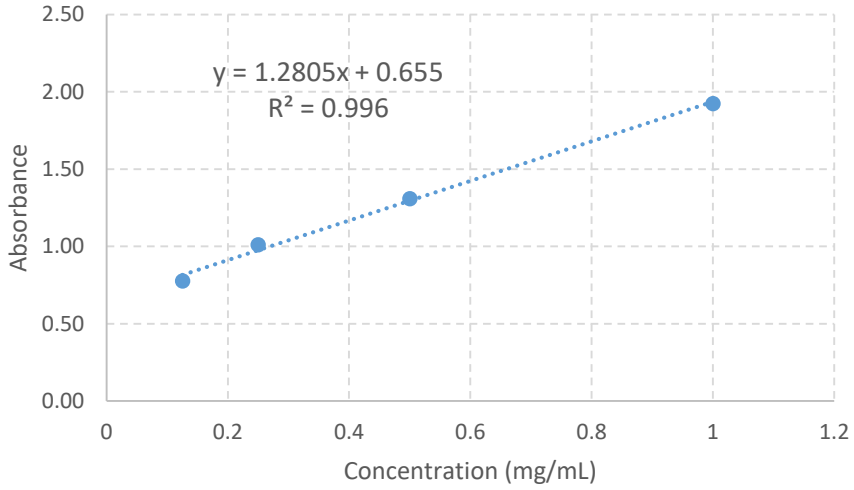


Figure 1. Protein standard curve with the regression equation and the coefficient of determination (R^2).

RESULTS AND DISCUSSION

Fermented food has gained a great popularity, particularly recently, due to its health benefits. This food is obtained by a controlled growth of microorganisms and conversion of food ingredients in enzymatic reactions (Dimidi et al., 2019). In the described experiments, the *B. subtilis* 3349 (ATCC-15245) strain was used, which is considered to be safe and of proven effectiveness, and which is able to grow and accumulate biomass fast (Ruiz Sella et al., 2021). The effective biomass growth is influenced by conditions such as the temperature, the time, and the oxygen availability, so the process was conducted in controlled conditions of humidity and temperature (75%, 37°C) for six days. Samples for test were collected every 24 hours, and then the influence of the process on macronutrients composition was examined.

The scope of tests covered determination of 37 fatty acids, with the number of carbon atoms ranging from 4 to 24, including saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Among unsaturated fatty acids, the study focused on three acids from the omega-3 group, three from the omega-6 group, and one from the omega-9 group. The results of conducted tests are shown in Tables 1–5.

Table 1. Profile of fatty acids in sunflower seeds.

Symbol	Acid name/Common name	Fermentation time [h]						
		0	24	48	72	96	120	144
C14:0	tetradecenoic acid, myristic acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C16:0	hexadecanoic acid, palmitic acid	5.0	4.9	4.9	4.6	4.7	4.7	4.5
C16:1	hexadecenoic acid, palmitoleic acid	0.2	0.2	0.2	0.2	0.2	0.2	0.2
C17:0	heptadecanoic acid, margaric acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C17:1	heptadecenoic acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C18:0	octadecanoic acid, stearic acid	4.6	4.6	4.4	4.2	4.2	4.2	4.3
C18:1, omega-9, cis, oleinowy	elaidic acid and oleic acid, (E)-octadec-9-enoic acid and (9Z)-octadec-9-enoic acid	18.7	19.5	17.7	17,8	19,6	19,6	17,1
C18:2, cis, omega-6	(9Z,12Z)-octadeca-9,12-dienoic acid, linoleic acid	67.0	66.6	68.1	68.7	66.9	67.0	69.3
C20:0	eicosanoic acid, arachidic acid	0.6	0.5	0.5	0.6	0.5	0.5	0.6
C20:1	cis-11-eicosenoic acid	0.3	0.3	0.3	0.3	0.3	0.3	0.3
C20:2	cis-11,14-eicosadienoic acid	0.1	0.1	0.2	0.1	0.2	0.2	0.1
C20:3, omega-6	dihomo-γ-linolenic acid, (8Z,11Z,14Z)-icosa-8,11,14-trienoic acid	0.5	0.5	0.5	0.6	0.5	0.5	0.6
C20:5, omega-3, EPA	eicosapentaenoic acid, (5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenoic acid	0.2	0.3	0.2	0.2	0.3	0.2	0.2
C22:0	docosanoic acid, behenic acid	1.6	1.4	1.5	1.5	1.5	1.4	1.6
C22:1	cis-13-docosenoic acid, erucate acid	0.0	0.0	0.2	0.0	0.0	0.0	0.0
C22:2	cis-13,16-docosadienoic acid	0.1	0.1	0.1	0.1	0.1	0.0	0.1
C23:0	tricosanoic acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C24:0	lignoceric acid	0.7	0.6	0.6	0.6	0.6	0.6	0.6

Table 2. Fatty acid profile in mung beans.

Symbol	Acid name/Common name	Fermentation time [h]						
		0	24	48	72	96	120	144
C16:0	hexadecanoic acid, palmitic acid	17.3	18.8	17.3	17.3	16.0	15.1	14.6
C16:1	hexadecenoic acid, palmitoleic acid	0.3	0.3	0.2	0.2	0.2	0.2	0.2
C17:0	heptadecanoic acid, margaric acid	0.3	0.3	0.3	0.3	0.3	0.2	0.2
C17:1	heptadecenoic acid	0.4	0.4	0.3	0.4	0.3	0.2	0.2
C18:0	octadecanoic acid, stearic acid	2.9	3.1	2.4	2.5	2.5	2.4	2.7
C18:1, omega-9, cis, oleinowy	elaidic acid and oleic acid, (E)-octadec-9-enoic acid and (9Z)-octadec-9-enoic acid	6.8	6.1	6.7	7.0	8.3	5.9	8.2
C18:2, cis, omega-6	(9Z,12Z)-octadeca-9,12-dienoic acid, linoleic acid	38.3	34.3	34.9	34.9	33.3	38.9	35.4
C18:3, omega-6, GLA	gamma-linolenic acid, (6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid	0.3	0.5	0.4	0.4	0.4	0.3	0.3

C18:3, omega-3, ALA	alpha-linolenic acid, (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid	25.0	26.9	29.8	29.8	31.6	31.2	32.4
C20:0	eicosanoic acid, arachidic acid	1.1	1.3	1.0	1.0	1.0	0.8	0.8
C20:1	cis-11-eicosenoic acid	0.6	0.6	0.5	0.5	0.5	0.4	0.4
C20:2	cis-11,14-eicosadienoic acid	0.6	0.6	0.5	0.5	0.5	0.3	0.4
C20:3, omega-3	cis,cis,cis-8,11,14-eicosatrienoic acid, dihomo-γ-linolenic acid	0.4	0.5	0.4	0.4	0.4	0.2	0.3
C20:3, omega-6	dihomo-γ-linolenic acid (8Z,11Z,14Z)-icosa-8,11,14-trienoic acid	0.5	0.5	0.4	0.4	0.4	0.3	0.3
C20:5, omega-3, EPA	eicosapentaenoic acid, (5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenoic acid	0.4	0.4	0.3	0.3	0.3	0.2	0.2
C21:0	heneicosanoic acid	0.3	0.3	0.3	0.3	0.3	0.2	0.2
C22:0	docosanoic acid. behenic acid	1.7	1.9	1.5	1.5	1.3	1.2	1.2
C22:1	cis-13-docosenoic acid, erucate acid	0.4	0.4	0.3	0.3	0.3	0.2	0.2
C22:2	cis-13.16-docosadienoic acid	0.2	0.2	0.2	0.1	0.2	0.1	0.1
C23:0	tricosanoic acid	0.5	0.5	0.4	0.4	0.4	0.3	0.3
C24:0	lignoceric acid	1.5	1.7	1.4	1.2	1.3	1.0	1.1

Table 3. Fatty acid profile of soybeans.

Symbol	**Acid name/Common name**	**Fermentation time [h]**						
		0	**24**	**48**	**72**	**96**	**120**	**144**
C10:0	decanoic acid. capric acid	0.2	0.0	0.0	0.0	0.0	0.0	0.0
C16:0	hexadecanoic acid, palmitic acid	8.1	6.9	6.4	6.2	5.8	5.3	5.3
C16:1	hexadecenoic acid, palmitoleic acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C17:0	heptadecanoic acid, margaric acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C17:1	heptadecenoic acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C18:0	octadecanoic acid, stearic acid	3.6	3.5	3.3	3.4	3.2	3.2	2.9
C18:1, omega-9, cis, oleinowy	elaidic acid and oleic acid, (E)-octadec-9-enoic acid and (9Z)-octadec-9-enoic acid	17.0	16.1	15.6	14.8	14.7	13.7	14.2
C18:2, cis, omega -6	(9Z,12Z)-octadeca-9,12-dienoic acid. linoleic acid	53.1	49.0	47.9	50.0	44.0	45.7	43.5
C18:3, omega-6, GLA	gamma-linolenic acid, (6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid	0.1	0.1	0.0	0.0	0.0	0.0	0.0
C18:3, omega-3, ALA	alpha-linolenic acid, (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid	15.8	22.4	24.7	23.7	30.3	30.1	32.0
C20:0	eicosanoic acid, arachidic acid	0.3	0.3	0.3	0.3	0.3	0.3	0.3
C20:1	cis-11-eicosenoic acid	0.2	0.2	0.2	0.2	0.2	0.2	0.2
C20:2	cis-11,14-eicosadienoic acid	0.2	0.1	0.1	0.1	0.1	0.1	0.1
C20:3, omega-3	cis,cis,cis-8,11,14-eicosatrienoic acid, dihomo-γ-linolenic acid	0.1	0.1	0.1	0.0	0.0	0.0	0.0
C20:3, omega-6	dihomo-γ-linolenic acid, (8Z,11Z,14Z)-icosa-8,11,14-trienoic acid	0.2	0.2	0.2	0.2	0.2	0.2	0.2

C20:5, omega-3, EPA	eicosapentaenoic acid, (5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenoic acid	0.1	0.1	0.1	0.1	0.1	0.0	0.1
C21:0	heneicosanoic acid	0.1	0.1	0.0	0.0	0.0	0.0	0.0
C22:0	docosanoic acid, behenic acid	0.4	0.4	0.4	0.4	0.4	0.4	0.4
C22:1	cis-13-docosenoic acid, erucate acid	0.1	0.0	0.0	0.0	0.0	0.0	0.0
C23:0	tricosanoic acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C24:0	lignoceric acid	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Table 4. Fatty acid profile of green lentils.

Symbol	**Acid name/Common name**	**Fermentation time [h]**						
		0	**24**	**48**	**72**	**96**	**120**	**144**
C16:0	hexadecanoic acid, palmitic acid	12.4	13.7	13.7	13.0	14.4	13.8	13.5
C16:1	hexadecenoic acid, palmitoleic acid	0.4	0.4	0.3	0.4	0.4	0.3	0.3
C17:0	heptadecanoic acid, margaric acid	0.3	0.3	0.3	0.3	0.3	0.3	0.3
C17:1	heptadecenoic acid	0.5	0.5	0.5	0.5	0.5	0.4	0.5
C18:0	octadecanoic acid, stearic acid	1.7	1.8	1.8	1.8	1.9	1.8	2.0
C18:1, omega-9, cis, oleinowy	elaidic acid and oleic acid, (E)-octadec-9-enoic acid and (9Z)-octadec-9-enoic acid	11.3	11.0	10.7	7.3	8.3	7.7	7.4
C18:2 ,cis, omega-6	(9Z,12Z)-octadeca-9,12-dienoic acid, linoleic acid	44.2	47.5	48.6	46.3	49.9	52.9	46.8
C18:3, omega-6, GLA	gamma-linolenic acid, (6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid	0.4	0.5	0.6	0.6	0.5	0.4	0.5
C18:3, omega-3, ALA	alpha-linolenic acid, (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid	18.5	14.8	14.8	20.4	14.3	14.0	19.8
C20:0	eicosanoic acid, arachidic acid	1.2	1.1	1.0	1.1	1.1	1.0	1.1
C20:1	cis-11-eicosenoic acid	1.1	1.1	1.1	1.1	1.2	1.0	1.2
C20:2	cis-11,14-eicosadienoic acid	0.8	0.7	0.7	0.8	0.7	0.6	0.7
C20:3, omega-3	cis.cis.cis-8,11,14-eicosatrienoic acid, dihomo-γ-linolenic acid	0.6	0.5	0.5	0.5	0.5	0.4	0.4
C20:3, omega-6	dihomo-γ-linolenic acid, (8Z,11Z,14Z)-icosa-8,11,14-trienoic acid	0.6	0.5	0.5	0.5	0.5	0.5	0.5
C20:5, omega-3, EPA	eicosapentaenoic acid, (5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenoic acid	0.5	0.4	0.4	0.4	0.4	0.4	0.4
C21:0	heneicosanoic acid	0.4	0.4	0.4	0.4	0.4	0.4	0.4
C22:0	docosanoic acid. behenic acid	1.7	1.6	1.5	1.6	1.6	1.4	1.5
C22:1	cis-13-docosenoic acid, erucate acid	0.5	0.5	0.4	0.5	0.5	0.4	0.4
C22:2	cis-13,16-docosadienoic acid	0.4	0.3	0.3	0.4	0.4	0.4	0.3
C23:0	tricosanoic acid	0.5	0.5	0.4	0.4	0.4	0.4	0.4
C24:0	lignoceric acid	1.6	1.5	1.3	1.5	1.5	1.2	1.3

Table 5. Fatty acid profile of field beans.

Symbol	Acid name/Common name	Fermentation time [h]						
		0	**24**	**48**	**72**	**96**	**120**	**144**
C10:0	decanoic acid. capric acid	1.0	0.4	0.0	0.2	0.0	0.0	0.0
C16:0	hexadecanoic Aid, palmitic acid	10.7	10.5	10.6	10.9	10.9	11.7	10.3
C16:1	hexadecenoic acid, palmitoleic acid	0.4	0.5	0.3	0.3	0.3	0.3	0.2
C17:0	heptadecanoic acid, margaric acid	0.3	0.3	0.3	0.3	0.3	0.3	0.2
C17:1	heptadecenoic acid	0.5	0.5	0.4	0.4	0.5	0.4	0.3
C18:0	octadecanoic acid. stearic acid	2.7	2.6	2.7	2.5	2.4	2.5	3.1
C18:, omega-9, cis, oleinowy	elaidic acid and oleic acid, (E)-octadec-9-enoic acid and (9Z)-octadec-9-enoic acid	8.3	7.2	5.9	7.4	5.9	6.2	4.7
C18:2, cis, omega-6	(9Z,12Z)-octadeca-9,12-dienoic acid, linoleic acid	46.8	47.8	51.3	49.2	51.0	48.6	55.2
C18:3, omega-6, GLA	gamma-linolenic acid, (6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid	0.4	0.5	0.4	0.4	0.4	0.4	0.4
C18:3, omega-3, ALA	alpha-linolenic acid, (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid	19.8	20.4	20.7	20.9	20.9	23.9	20.2
C20:0	eicosanoic acid, arachidic acid	1.1	1.1	0.9	0.9	0.9	0.8	0.7
C20:1	cis-11-eicosenoic acid	0.8	0.9	0.7	0.7	0.7	0.6	0.6
C20:2	cis-11,14-eicosadienoic acid	0.7	0.8	0.6	0.6	0.6	0.5	0.4
C20:3, omega-3	cis.cis.cis-8,11,14-eicosatrienoic acid, dihomo-γ-linolenic acid	0.5	0.5	0.4	0.4	0.4	0.3	0.3
C20:3, omega-6	dihomo-γ-linolenic acid, (8Z,11Z,14Z)-icosa-8,11,14-trienoic acid	0.6	0.7	0.5	0.5	0.5	0.4	0.5
C20:5, omega-3, EPA	eicosapentaenoic acid, (5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenoic acid	0.5	0.5	0.4	0.4	0.4	0.3	0.2
C21:0	heneicosanoic acid	0.4	0.4	0.3	0.3	0.3	0.2	0.2
C22:0	docosanoic acid, behenic acid	1.5	1.5	1.3	1.2	1.2	0.9	0.9
C22:1	cis-13-docosenoic acid, erucate acid	0.5	0.5	0.4	0.4	0.4	0.3	0.2
C22:2	cis-13,16-docosadienoic acid	0.2	0.2	0.2	0.2	0.2	0.1	0.1
C23:0	tricosanoic acid	0.4	0.4	0.3	0.3	0.4	0.3	0.2
C24:0	lignoceric acid	1.5	1.5	1.2	1.2	1.2	0.9	0.8

The studied plant matrices proved to be a good source of fatty acids. The sunflower contained the highest quantity of omega-6 polyunsaturated fatty acids with 18 carbon atoms and two double bonds in the cis (C18:2) configuration - the highest increase in the content of that acid (69.3%) was noted in the 144th hour of the fermentation. It can also be noticed that as the fermentation of the sunflower seeds by B. *subtilis* proceeded, the concentration of omega-9 acids with 18 carbon atoms and one double bond also increased - after 96 hours of the process it amounted to 19.6%. However, no omega-3 and omega-6 C18:3 fatty acids and fatty acids containing from 4 to 13 and 15 and 21 carbon atoms in their chain were found in the sunflower seeds (Table 1). In the case of the mung bean fermentation, also the highest concentration of C18:2 cis acids is observed, which reaches its peak of 38.9% in the 120th hour of the process. Furthermore, C18:3, omega-3 acid ALA was found in the mung bean, in the quantity ranging from 25.0% (at the beginning of the process) to 32.4% (on the last day of fermentation), as well as C18:1, omega-9, cis oleic acid, ranging from 6.8 to 8.2% during the process (Table 2). The following acids were present in the highest quantities in the fatty acids profile of the soy beans before fermentation: C18:2, cis, omega-6 - 53,1%; C18:1, omega-9, cis, oleic - 17%; C18:3, omega-3, ALA – 15.8%; C16:0 – 8.1%; and C18:0 - 3.6% (Table 3). The C18:3, omega-3 acid, ALA, was present in the highest quantities (32.0%) after 144 hours of fermentation. The percentage content of C18:2, cis, omega-6 acid and of C18:1, omega-9, cis, oleic acid decreased slightly until the end of the process. In the soy beans, the remaining fatty acids represented 2.4% before fermentation, and 2.1% after fermentation. The following acids were present in the highest quantities in the fatty acids profile of the pea before fermentation: C18:2, cis, omega-6 - 46.8%; C18:3, omega-3, ALA - 19.8%; C16:0 - 10.7%; C18:1, omega-9, cis, oleic - 8.3%; and C18:0 - 2.7%. The content of the C18:3, omega-3 acid, ALA reached its peak (23.9%) after 120 hours of fermentation. The C18:1, omega-9, cis, oleic acid was presented at the highest quantity (7.4%) after 72 hours of the process. The C18:2, cis, omega-6 acid reached its peak (48.6%) in the fermented pea seeds after 120 hours of the process. The remaining acids represented 11.7% and 6.5% before and after the pea fermentation, respectively (Table 5). For the lentils, the best efficiency for the linoleic acid (52.9%) was achieved in the 120th hour of fermentation, and for the C18:3, omega-3 acid, ALA, it was reached at the end of the process, i.e., after 144 hours of fermentation (Table 4). The content of the C18:1 omega-9, cis, oleic acid decreased during fermentation, to reach 7.5%. In the mung beans, the lentils, and the peas, the arachidic acid C16:0 was found, representing 15%, 14% and 10%, respectively. In two matrices, the quantity of that acid decreased slightly during processing (Tables 2, 4, and 5). Furthermore, palmitic (C16:1), erucic (C22:1) and tricosanoic (23:0) acids were detected in the analyses, but they were only present at trace levels (below 1%). Low levels of arachidic (20:0) and cis-11-eicosenoic (C20:1) acids were also found in the analysed samples.

In the fermented plant products, PUFA represented the largest percentage content of all fatty acids, amounting to 73.6% in the soy, 67.7 % in the lentils, and 67.3% in the pea and the sunflower. SAFA were the second group of acids in the fermented material in terms of their quantity - representing 24.0 % for the mung bean, the pea, and the sunflower, and 10.7% for the soy, while the largest quantities of MUFA were found in the soy (15.6%), the lentils (11.8%), and in the mung bean, the pea, and the sunflower (8.7%). The total content of the fatty acids in the studied seeds is shown in Figure 2.

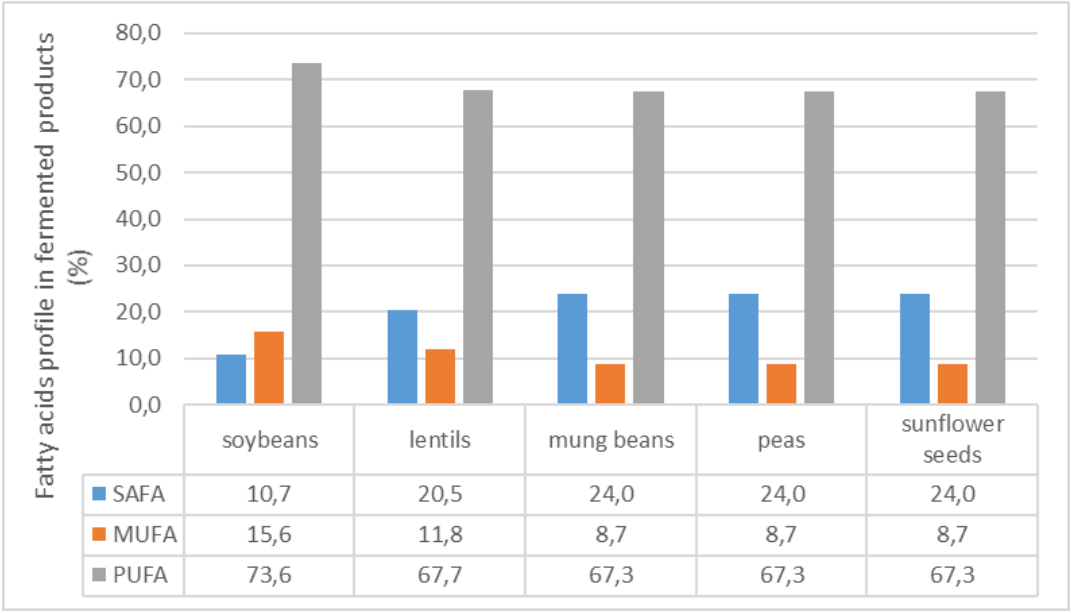
The studies of Khrisanapant et al. (2019) demonstrated the high content of EFA, with 47.3% in the lentils, and 71% in the black bean. In studies of Grel et al. (2017), aiming at determining the fatty acids profile of the selected pulses, it was demonstrated that the highest content of ALA (C18:3, n-3) was noted in the lentils, the kidney bean, and the pea, amounting to (13.8 g per 100 g of fat), (11.9 g per 100 g of fat) and (10.4 g per 100 g of fat), respectively. Amounts of PUFA found in the black bean (71%) were higher than in the fermented pea (67.3%) (Grela et al., 2017).

The conducted analyses of fatty acids were consistent with results obtained by Cho et al., (2017), who also noted the highest content of the linoleic acids (50.84%) in the soy beans (Cho et al., 2017). The total content of unsaturated fatty acids in dry soy beans corresponded to 78.68% of all fatty acids, and the highest quantity (50.23%) was noted for the fatty acid C18:2 (Pattee et al., 1983).

In the conducted study, we demonstrated that prolonging of the fermentation time to 120 hours or 144 hours leads to the increases in the polyunsaturated fatty acids content in all studied plant matrices. Similar conclusions were also reached by other researchers, who discovered that presence of polyunsaturated fatty acids and the prolonged fermentation time positively influence the process effectiveness (González-Pérez & Vereijken, 2007). Park et al., (2010), on the other hand, observed the increase of the oleic (C18:1),

linoleic (C18:2) and alpha-linolenic (C18:3) acids during 50 hours of fermentation of plant material.
Kim et al., (2012) also demonstrated that prolonging the fermentation time to 48 hours had a positive influence on changes in the fatty acids profile. The prevalence of these acids additionally improves the nutritional value of studied plant matrices. It was proven that they have an important role in reduction of cholesterol blood levels (Petraru et al., 2021). Furthermore, they are necessary to ensure normal
In the conducted studies, it was found that the content of polyunsaturated fatty acids in seeds is lower than in their fermented equivalents. As it is well known, the human body cannot synthesise them and they must be supplied with food, so the fermented food represents an excellent alternative to the functional food. It can be easily used in people with chronic diseases, as it has a proven positive effect on the body.
Pulses are a rich source of dietary proteins of an advantageous amino acid composition, especially exogenous amino acids, which the body cannot produce itself. Leucine, lysine, phenylalanine, and threoninee dominate in this group. These plants are also rich in minerals and vitamins (Hou et al., 2019). Certain seeds, like the soy bean, the mung bean, or the pea, also contain undesirable substances that may hinder absorption of nutrients. They include protease and trypsin inhibitors, lectin, urease, and tannins or oligosaccharides in smaller quantities (Vagadia et al., 2017). These compounds cross-link with proteins, making them less susceptible to enzymes during digestion (Çabuk et al., 2018). Numerous scientific evidence shows that antinutrients levels can be reduced or eliminated using various processing methods like cooking or fermentation. In particular, fermentation with *B. subtilis* may not only influence physical and chemical changes in seeds, but also their digestibility (Zhang et al., 2020). Kim et al., (2012) analysed the metabolic profile of the fermented soy beans using the GC-MS technique, and the process was conducted using a strain of B. *subtilis*. They demonstrated that fermentation may improve digestibility of proteins, when compared with unfermented seeds. Furthermore, they have proven that separation of metabolites was significantly influenced by the duration of the process, and not only by the bacteria strain used. Probably, this resulted from non-specific enzymatic activity of microorganisms in relation to seed proteins (Cho et al., 2017).
In the conducted experiments, a drop in the protein content was demonstrated in the studied plant material. Detailed data on the protein content in specific fermented matrices is shown in Table 6 and Figure 3.

body functions (Zia-Ul-Haq et al., 2008). Certain literature data show that the fermentation process with B. *subtilis* contributes to reduction of the lipid content in the seeds, and this may result from the strong lipolytic activity of B. *subtilis* (Shrestha et al., 2013). Anwar et al., (2007) in their studies demonstrated a drop in the lipid content in the seeds during fermentation, ranging from 22.8 - 24.5 and 14.8 - 17.6 mg/100 g of dry weight (d.w.).



	soybeans	lentils	mung beans	peas	sunflower seeds
SAFA	10,7	20,5	24,0	24,0	24,0
MUFA	15,6	11,8	8,7	8,7	8,7
PUFA	73,6	67,7	67,3	67,3	67,3

Figure 2. The percentage of fatty acids in fermented seeds.

The greatest drop in the protein content during fermentation was observed for the pea seeds and amounted to 9.676 (mg/100 g d.w.) and in the mung beans, reaching 6.137 (mg/100 g d.w.). The lowest drop in the protein content was observed in the lentil seeds and amounted to 1.643 (mg/100 g d.w.). In the sunflower seeds, the protein content also went down during fermentation, from 26.76 mg/100 g d.w. to 23.35 mg/100 g d.w. during 144 hours of the process. The similar drop in the content of this ingredient was observed in the soy. The initial protein content in these seeds was 46.179 mg/100 g d.w., and on the last day of fermentation it went down to 42.052 mg/100 g d.w. The details are provided in Table 6.

This reduction in the protein content during the fermentation process was also observed by other researchers. In the study by Wang et al. (2021) it was demonstrated that B. *subtilis* during its growth process is able to produce extracellular enzymes that digest nutrients, like proteins and carbohydrates. During fermentation of millet bran, also a drop in the protein content was observed, from 4.3% in unfermented seeds to 2.1% in seeds after fermentation (Chu et al., 2019). However, Ruan et al. (2020) got different results, and during fermentation of the soy beans they noticed that their peptide content increased with its duration. Similar results were obtained by Baek et al. (2010), who demonstrated an increased share of amino acids during 72 hours of fermentation. Tao et al. (2017), conducting lactic acid fermentation using the B1-6 strain of *Lactobacillus plantarum*, demonstrated that the protein level in the product was 33.1 g/100 g before fermentation, and with the duration of the process it increased to 34.19 g/100 g after 17 hours (Tao et al., 2017). An increase in the peptides content was also noted during fermentation of the black soy beans after 24 hours of the process (Zhu et al., 2008). This increase in the protein content may be attributed to the dry matter loss during fermentation following hydrolysis and metabolism of carbohydrates as an energy source for microorganisms (Niu et al., 2013). Numerous studies prove that the protein production during fermentation is influenced by the composition of the fermentation medium. Selection of an appropriate substrate for solid state fermentation is of great significance for successful process. In the conducted experiments, the process was conducted using the LB MIX medium, which is a good source of carbon, vitamins, minerals, and proteins required for growth of microorganisms (Mukhtar & Haq, 2013). These components also participate in the B. *subtilis* respiration (Chen et al., 2009).

Proteins belong to factors that may reduce the fermentation rate (Li et al., 2019). This process may involve many biochemical changes. B. *subtilis* proteases may influence decomposition of complex proteins to simple ones (Cuevas-Rodríguez et al., 2004). Furthermore, microorganisms use amino acids, and this may lead to a decrease in the total protein content of the seeds. Fermentation may also facilitate decomposition of plant cell walls, and contribute to release of bioactive compounds (Pranoto et al., 2013). In their studies, Torino et al., 2013, suggested that bioactive peptides produced by *B. subtilis* proteases may improve biological properties of fermented products. Additionally, fermentation with *B. subtilis* may be used as an effective strategy to strengthen proteolysis and the anti-oxidative effect, while the fermented seeds can become functional food (Li & Wang et al., 2021).

Table 6. Content of protein in fermented seeds.

Fermentation time [h]	**Protein concentration [mg/100 g d. w.]**				
	Sunflower	**mung beans**	**Soybeans**	**Green lentil**	**Field beans**
0	26.796	24.645	46.179	14.120	32.951
24	25.234	22.259	44.129	13.258	26.415
48	24.937	21.183	43.611	12.439	25.820
72	24.196	20.994	43.313	12.592	26.314
96	23.977	20.917	42.987	12.829	24.581
120	23.549	20.967	42.311	12.775	24.090
144	23.352	18.508	42.052	12.477	23.275

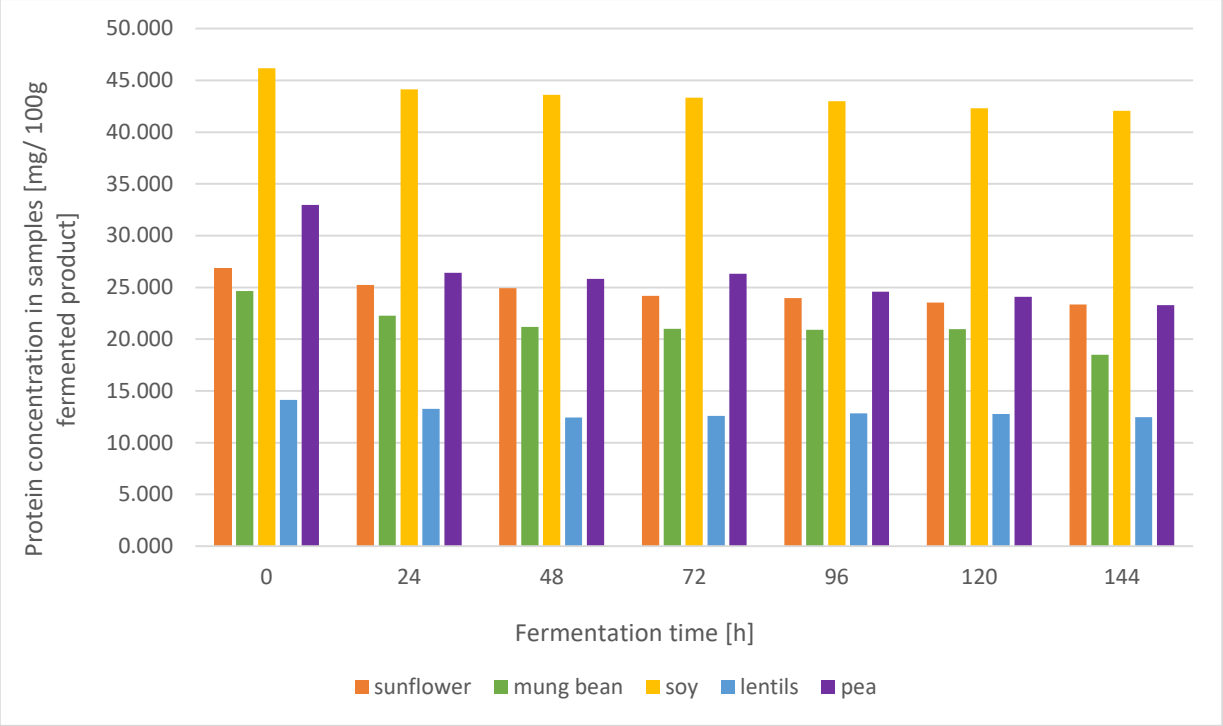


Figure 3. Protein content in plant material during the fermentation process.

CONCLUSIONS

On the basis of obtained results it can be observed that fermentation of the studied pulse seeds with the use of *B. subtilis* enriches these products with valuable nutrients. In the conducted experiment, it was observed that when fermentation of the studied seeds (depending on a type of the studied plant material) was prolonged to 144 hours, the content of n-3 and n-6 polyunsaturated fatty acids (PUFA) increased. After 144 hours of the fermentation process, the omega-6 linoleic acid was a dominating fatty acid in the studied plant matrices. Furthermore, the C18:2, cis, omega-6 fatty acid also had the highest percentage share of all fatty acids before fermentation, and after each completed day of fermentation. Of the studied seeds, the highest levels of the linoleic acid were obtained during fermentation of the sunflower, the lentil, and the pea seeds, changing from 7.0% to 69.3%; from 44.2% to 46.8% and from 46.8% to 55.2%, respectively, when compared to baseline values.

This study showed that the protein levels in the studied plant matrices decrease with the *B. subtilis* fermentation time. The highest drop in the protein content was observed for the pea (by 9.676 mg/100 g of d.w.) and the mung bean (by 6.137 mg/100 g of d.w.), while the lowest decrease in the protein content was found in the lentil seeds (by 1.643 mg/100 g of d.w.), versus baseline levels.

The controlled fermentation of plant matrices is a crucial process for obtaining products with improved nutritional properties, which can be successfully used in production of functional food. Furthermore, due to high and balanced levels of omega-3 and omega-6 acids in fermented pulse seeds, they can replace fish in a diet of vegetarians and vegans.

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