

## Formulation of synbiotic soy-based food product with antihypertensive potential

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### Abstract

The specific aims of this study were to select the favorable prebiotic for *L. casei*-01 as well as the suitable inoculum of the probiotic for fermented soy with ACE-inhibitory potential. For that purpose the metabolic activity of *L. casei*-01 in soymilk supplemented with (1.5% w/v) prebiotics Fructooligosaccharide (FOS) or oligofructose enriched inulin (Synergy 1) was assessed. The evaluated parameters were: pH, viable cell counts, proteolysis, organic acid production and inhibition of angiotensin converting enzyme activity (IACE). The cell growth of *L. casei*-01 reached the recommended therapeutic level of  $9.58 \pm 0.035 \log \text{cfu mL}^{-1}$  for low inoculum samples (0.005 and 0.01%w/v) and  $11.543 \pm 0.13 \log \text{cfu mL}^{-1}$  for high inoculum samples (0.075 and 0.1%w/v) regardless of the prebiotic used. The lower pH during fermentation, faster cell growth and superior proteolysis in Synergy 1 samples indicated better utilization of that prebiotic vs. FOS. The hydrolysis depended on the prebiotic used, showing higher values in Synergy 1 samples. The faster proteolysis was confirmed by SDS-PAGE electrophoresis. The Mw of polypeptides in the synbiotic end-products were lower than 30kD. The observed values for inhibition of ACE activity were app. 71, 74, 77 and 78% for inoculum rates of 0.005, 0.01, 0.075 and 0.1% w/v, respectively.

Based on the results obtained in our study, the prebiotic Synergy 1 (1.5% w/v) and *L. casei*-01 at inoculum of 0.01% w/v for low dose and 0.075% w/v for high dose were considered more favorable for the production of synbiotic soy drink with antihypertensive potential.

**Key words:** *L. casei*, Synergy 1, FOS, soy beverage, ACE inhibition

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### Introduction

Awareness of complex relationship between food and health has challenged researches to design functional food, food that not only provide basic nutrition but also

has health benefit effects (Gibson and Rastall, 2004). High prevalence of hypertension, one of the major risk factors for coronary heart disease, indicates an urgent need for prevention and choice of therapy. From the standpoint of preventive medicine, soybean-based foods are of great interest because of the evidence that consumption of soy proteins 25 g per day can lower the risk of cardiovascular dis-

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ease, an indication approved by the U. S. Food and Drug Administration (1999).

Many improvements have been implemented in the processing and methods of preparation of soymilk to improve functionality and usability of soy proteins, among which fermentation of soymilk with probiotic bacteria (Favaro Trindade et al., 2001; Lopez-Fandino et al., 2006).

Soy-milk-derived bioactive peptides during fermentation have many beneficial properties, including prevention or delay in the onset of hypertension (Wang and Mejia, 2005; Liu et al., 2011). Well-known mechanism of action of these peptides is based on the inhibition of angiotensin-I converting enzyme (ACE) (Vermeirssen et al., 2004). ACE inhibitory activity of milk products can be enhanced by using the highly proteolytic lactic acid bacteria (LAB). According to Donkor et al. (2005) the use of probiotic strains such as *L. paracasei*, as a part of starter culture in fermented soymilk resulted in a considerable increase in ACE inhibitory activity *in vitro* compared with the control produced by yogurt culture only (Donkor et al., 2005).

Probiotics differ in their ability to ferment the bioactive fibers. For the production of fermented milk with hypotensive and/or ACE-inhibitory activity, various LAB species have been used, including probiotic *Lactobacillus paracasei* (Fuglsang et al., 2003; Fitzgerald et al., 2006).

Nowadays, to improve therapeutic effects, dairy foods usually contain probiotics along with prebiotics. Inulin and fructooligosaccharide (FOS) are the premium prebiotics used as safe ingredient supplements to fermented milk (Oliveira et al., 2012), with protective effect on the lactic acid bacteria by stimulating their survival and activity of the end-product during storage (Donkor et al., 2006). Furthermore, they enhance the proteolytic activity and ACE-inhibition activity of lactobacilli in fermented soymilk (Yeo and Liong., 2010).

The mixture of probiotics and prebiotics is termed a synbiotic (Gibson and Roberfroid., 1995). The ability of microorganisms to utilize prebiotics is strain- and substrate-specific. Because of that, one of the essentials for good synbiotic formulation is a proper choice of pro- and pre mixture.

The synbiotic mixture of probiotics and prebiotics as well as the composition of a delivery matrix are responsible for variations in the amounts of organic acids, bioactive peptides production and bacterial count in the end-product of fermented functional food. The proteolytic activity should not destroy the product, but it should be able to produce the bioactive peptides in sufficient amount and desired activities. Moreover, it is important for probiotic bacteria to survive the food processing and storage in amount above therapeutic level ranging from  $10^6$  to over  $10^7$  or  $10^8$  cfu mL<sup>-1</sup> (Kurmann and Rasic., 1991; Lourens-Hattingh and Viljeon., 2001).

The aim of the study was to prepare fermented soymilk supplemented with prebiotic, in which probiotic viability would be increased, and thus its proteolytic and/or ACE in-

hibitory activity enhanced. The effects of prebiotics, FOS and oligofructose-enriched inulin and different inoculum rates of *L. casei-01*, on the metabolic activity and bioactivity of probiotic-fermented soymilk was evaluated *in vitro* by determination of pH, organic acid production, proteolytic activity and ACE inhibition. In addition, peptide profile of the soy beverage was evaluated.

## Material and methods

### *Fermentation of soymilk with pro- and pre-biotic*

Two batches of 5 glass flasks, each containing 200 mL commercial (cholesterol, lactose and gluten free) soymilk (Valsoia Original – VALSOIA SpA, Italy) were supplemented with 1.5 % w/v prebiotic. Batch 1 was supplemented with oligofructose-enriched inulin (Synergy-1, Orafiti® Synergy 1, Orafiti-Rue L. Maréchal, Tienen, Belgium) and batch 2 with fructooligosaccharide (FOS, Sigma Aldrich Chemie GmbH, Germany). The mixtures were heat treated in a water bath at 90 °C for 30 min, then cooled to 37 °C and aseptically inoculated with *L. casei-01*, commercial name for *Lactobacillus paracasei sp paracasei* (FD-DVS/*Lactobacillus casei-01* nu-trish, Chr. Hansen, Hoersholm, Denmark), with different inoculum rates: 0.005% w/v (SN1 and FS1), 0.01% w/v (SN2 and FS2), 0.075% w/v (SN3 and FS3), 0.1% w/v (SN4 and FS4) and 1% w/v (SN5 and FS5). SN represents samples supplemented with Synergy 1 and FS samples with FOS. All batches were incubated at 37 °C until the required pH of 4.5 was reached; then the fermentation was terminated by cooling at 4 °C. At predetermined time points and at the end of fermentation the aliquots of 30 mL were taken from each bottle for determination of cell count, pH, proteolysis and organic acid production. The end-products were analyzed for ACE-inhibitory activity.

### *pH measurements*

Changes in pH were monitored during fermentation of soymilk at 0, 6, 12, 24, 36, 48, 54 h and at the end of fermentation using pH meter (Mettler Toledo Five Easy™ FE 20, Switzerland).

### *Enumeration of viable cells*

Serial dilutions of the samples made in saline water (0.9 % w/v NaCl, Alkaloid, Skopje, Republic of Macedonia) were spread onto MRS agar plates (de Man, Rogosa Sharpe agar, Oxoid, Basingstore, UK) and incubated for 48 h at 37 °C in anaerobic conditions. All dilutions were plated in triplicate. Enumeration was performed counting the plates with 25-250 colonies. Mean numbers from two different dilutions were used, and results were expressed as log<sub>10</sub> colony forming units per milliliter of fermented milk (log<sub>10</sub> cfu mL<sup>-1</sup>).

### Determination of proteolytic activity

Proteolytic activity was determined as the difference between the free amino ( $\text{NH}_2$ ) groups in the fermented and unfermented (untreated) soymilk. The *o*-phthalaldehyde (OPA) method of Church et al. (1983) was used, with some modifications as reported by Donkor et al. (2005). Briefly, 3 mL of the sample with 3 mL 0.75N TCA ( $\text{CCl}_3\text{COOH}$ ) were vortexed for 1 min, centrifuged at 12 000 rpm, 15 min and afterwards the supernatants were filtered through 0.45  $\mu\text{m}$  membrane filter. The OPA reagent was prepared by mixing 40 mg of OPA (*o*-phthalaldehyde, P1378, Sigma Aldrich Chemie GmbH, Germany) freshly dissolved in 1 mL methanol ( $\text{CH}_3\text{OH}$ , Merck, KGaA Damstadt, Germany), 25 mL of 100 mM sodium tetraborate decahydrate ( $\text{Na}_2\text{B}_4\text{O}_7 \times 10\text{H}_2\text{O}$ , Alkaloid, Skopje, Republic of Macedonia), 2.5 mL 20% w/w sodium dodecyl sulfate (SDS,  $\text{NaC}_{12}\text{H}_{25}\text{SO}_4$ , Merck, KGaA Damstadt, Germany), 100  $\mu\text{L}$   $\beta$ -mercaptoethanol ( $\text{C}_2\text{H}_6\text{OS}$ , Merck, KGaA Damstadt, Germany) and deionized water to final volume of 50 mL. 150  $\mu\text{L}$  of filtered sample (supernatant) was mixed with 3 mL of OPA reagent and after 2 minutes at room temperature (20 °C) the absorbance was measured at 313 nm by a UV/Vis Spectrophotometer-Agilent 8453 (USA). The proteolytic activity was expressed as absorbance of free amino groups measured at 313 nm as a difference in absorbance between fermented and non-fermented samples.

### Analysis of peptide profile by SDS-PAG electrophoresis

To determine the proteolytic pattern of soy beverage, samples were centrifuged at 4 000 rpm for 20 min (Rotorfix 32A Hettich, Hettich Lab technology, Fohrenstrasse 12, D-78532 Tuttlingen, Germany). Then, the supernatants were separated, centrifuged again at 12 000 rpm for 20 min and filtered through 0.45  $\mu\text{m}$  membrane filter (MILLEX<sup>®</sup>-HP, Merck Millipore Ltd., Ireland). The protein transition to low molecular peptides was analyzed with horizontal (4-22 %) gradient gel sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAG electrophoresis) (Gorg et al., 1985). When electrophoresis was completed, the protein bands were stained with Coomassie Blue R-250 (Sigma -Aldrich) in methanol ( $\text{CH}_3\text{OH}$ , Merck, Germany) / water/acetic acid ( $\text{C}_2\text{H}_4\text{O}_2$ , Alkaloid, Skopje, Republic of Macedonia) and then destained in the same solvent. The fractions were identified by using low molecular mass standard mixture. The molecular weights (Mw) of different proteins were as follow: phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor 20.1 kDa and  $\alpha$ -lactalbumin 14.4 kDa (Pharmacia LKB, Biotechnology AB, Uppsala Sweden); and high molecular weight proteins as follows: myosin, 212 kDa;  $\alpha_2$ -macroglobulin, 170 kDa;  $\beta$ -galactosidase, 116 kDa; transferrin, 76 kDa; and glutamic dehydrogenase 53 kDa.

### Determination of produced organic acids in fermented soymilk

The amount of short chain organic acids was determined by high-performance liquid chromatography using a HPLC system apparatus equipped with an ultraviolet detector (Agilent Technologies 1200, Palo Alto, USA). The method used by Wang et al. (2003) was applied with certain modifications. In brief, to determine the concentrations of lactic and acetic acid, 2 mL of 0.5 M  $\text{H}_2\text{SO}_4$  were added to a 2-mL aliquot of the sample, thoroughly mixed for 30 s and centrifuged (12 000 rpm for 15 min). The obtained supernatants were filtered through a 0.45  $\mu\text{m}$  membrane filter (MILLEX<sup>®</sup>-HP, Merck Millipore Ltd., Ireland). Samples were loaded onto a thermostatically controlled reverse phase column (Discovery HS C 18, 250 mm x 4.6 mm, 5  $\mu\text{m}$ , Supelco Park, Bellefonte, PA, USA) set at 40 °C and eluted with 0.005 M  $\text{H}_2\text{SO}_4$  (Alkaloid, Skopje, Republic of Macedonia) at flow rate of 1 mL min<sup>-1</sup>. According to the method applied, the detection wavelength was 210 nm, while identification of lactic and acetic acids was done using their respective standards.

### Determination of ACE-inhibitory activity (IACE)

For the IACE (angiotensin-converting enzyme inhibition) assay the following chemicals were used: the substrate N-[3-(2-furyl)-acryloyl]-L-phenylalanine-glycyl-glycine (FAPGG,  $\text{C}_{20}\text{H}_{21}\text{N}_3\text{O}_6$ , No 7131 Mw 399.4 g mol<sup>-1</sup>; Sigma-Aldrich, Co. St Louis. Mo. USA), ACE control E (pure ACE from porcine kidney, Trinity Biotech, USA), and Tris buffer (2 hydroxymethyl-1,3-propanediol, No 108382, Mw 121,14 g mol<sup>-1</sup>; Merck, KGaA Damstadt, Germany).

Whey samples from the fermented soymilk were prepared by the following procedure: the end-products were centrifuged (4 000 rpm for 20 min at 4 °C) and the supernatants were adjusted to pH 8, centrifuged again at 12 000 rpm for 20 min (Biofuge Fresco Heraeus Instruments, USA) and filtered through 0.45  $\mu\text{m}$  membrane filter (MILLEX<sup>®</sup>-HP, Merck Millipore Ltd., Ireland).

Whey samples were analyzed by ACE inhibition assay using the method introduced by Tomovska et al. (2011), with slight modifications. Pure ACE from porcine kidney (ACE control E) for ACE solution was used (Vermeirssen et al., 2002). The reaction mixture consisted of a substrate (0.8 mmol L<sup>-1</sup> FAPGG in 50 mmol L<sup>-1</sup> Tris buffer, pH 8.2, chlorides 300 mmol L<sup>-1</sup>), ACE solution (ACE control E - freshly reconstituted with 1 mL deionized water according to the distributor), and sample (supernatant of fermented soymilk) in ratio 10 : 1 : 1. The reaction mixture was stirred by flipping the cuvette, which was then placed at 37°C. During incubation, the absorbance at 340 nm (with deionized water as a blank) was recorded for 45 min at exactly 5 min intervals. The absorbance was measured by the Ultrospec 6300pro UV/Visible Spectrophotometer (GE Healthcare, Fisher Scientific, UK Ltd). ACE activity was

expressed as the slope of the decrease in absorbance at 340 (ρA) over a linear interval of 45 min. The percentage of ACE inhibition (%IACE) was calculated from the ratio of the slope in the presence of inhibitor (sample) to the slope obtained in the absence of inhibitor (deionized water instead) using the following equation (Shalaby et al., 2006):

$$\text{ACE inhibition (\%IACE)} = [1 - (\rho A C - D / \rho A A - B)] \times 100$$

where A contained substrate solution, ACE solution and deionized water; B, substrate blank contained only substrate solution without ACE solution; C, sample (inhibitor) contained substrate solution, ACE solution and sample; D, sample blank contained substrate solution, sample and deionized water instead of ACE solution.

### Statistical analysis

The results obtained are presented as means±SD. Differences were determined by using multiple comparison tests: the ANOVA analyses and Tukey honest significant difference (HSD) test. Correlation analyses between parameters were also made (Statgraph for Windows 3.0). In all tests, a probability level of p<0.05 was used as a significant difference.

## Results and discussion

### Changes of pH and viability of *L. casei-01* during fermentation of prebiotic soymilk

pH changes, viable cell counts of *L. casei-01* and its metabolic activity were examined to evaluate the fermentation patterns of the prebiotics added.

The pH changes during fermentation at 37°C for all samples are shown in Fig. 1.

The initial pH of the *L. casei-01* and prebiotic supplemented soymilk, at time point 0, for all samples was 6.80 ± 0.01. Upon fermentation at 37°C for 30 to 62 h, depending on the inoculum rate (Table 1), it reduced by app. 34%, reaching a value of 4.50. Among the samples fermented with different inoculum rates, both Synergy 1 and FOS supplemented, difference in pH decline rate was observed. Significant difference (p<0.05) in pH decline rate was observed within the first 6 h of fermentation between the samples supplemented with low (0.005 and 0.01% w/v) and high (0.075, 0.1 and 1% w/v) inoculum rates. The high inoculum samples showed a continuous decrease in pH from the very beginning of fermentation, while in the low inoculum samples, a delay of 6 h was observed. Namely, six hours after inoculation, the pH value in the high inoculum samples decreased to

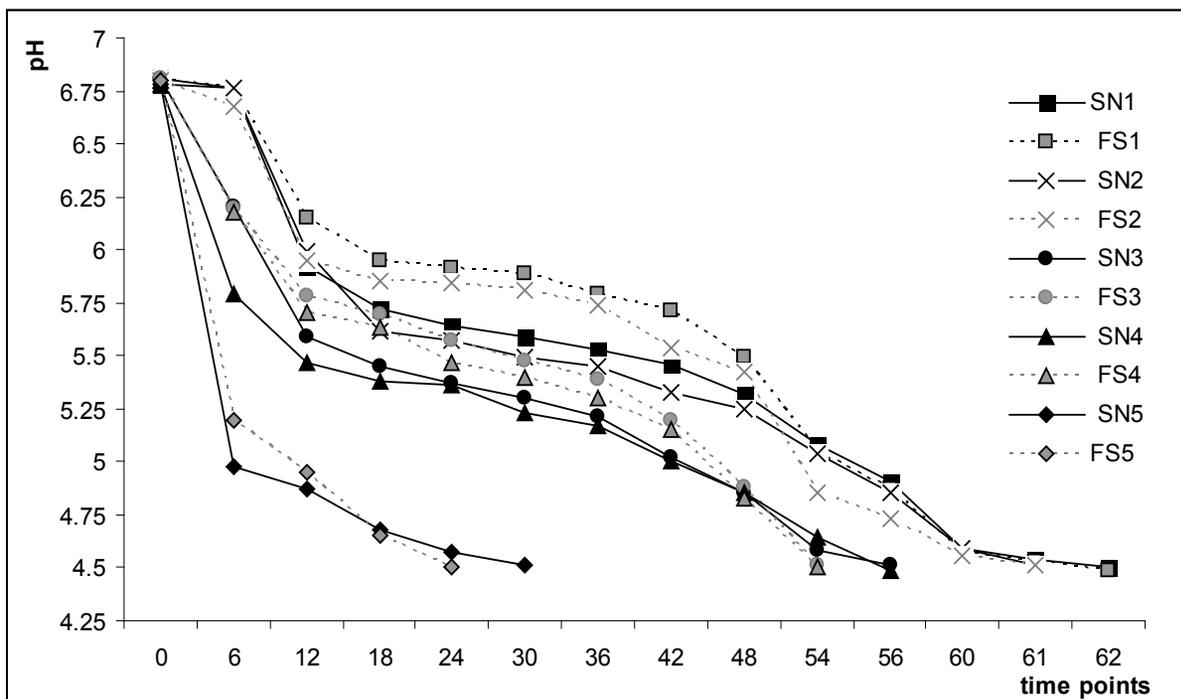


Fig. 1. pH changes during fermentation of Synergy 1 (SN) vs. FOS (FS) -supplemented soymilk by *L. casei-01*; Time points (hours). (Inoculum rate: 0.005%w/v, SN1 FS1; 0.01%w/v, SN2 FS2; 0.075%w/v SN3 FS3; 0.1%w/v, SN4 FS4; 1%w/v SN5 FS5).

Table 1. Viability and proteolytic activity of *L. casei*-01 in end-products

<i>L. casei</i> -01 Inoculum rate % w/v	Cell count (log <sub>10</sub> cfu mL <sup>-1</sup> )		Proteolytic activity (absorbance at 313 nm)	
	Synergy 1	FOS	Synergy 1	FOS
0.005*	9.59±0.03 <sup>a</sup>	9.54±0.09 <sup>a</sup>	0.54±0.02 <sup>a</sup>	0.31±0.01 <sup>d</sup>
0.01*	9.63±0.18 <sup>a</sup>	9.58±0.16 <sup>a</sup>	0.59±0.03 <sup>a</sup>	0.35±0.07 <sup>d</sup>
0.075 <sup>#</sup>	11.49±0.13 <sup>b</sup>	11.45±0.06 <sup>b</sup>	1.11±0.04 <sup>b</sup>	0.60±0.07 <sup>a</sup>
0.1 <sup>#</sup>	11.69±0.36 <sup>b</sup>	11.56±0.21 <sup>b</sup>	1.13±0.03 <sup>b</sup>	0.67±0.06 <sup>a</sup>
1 <sup>&amp;</sup>	10.51±0.34 <sup>c</sup>	10.25±0.28 <sup>c</sup>	3.09±0.14 <sup>c</sup>	N.D.

End-product was obtained after 62 h (\*), 56 h (#) and 30 h (&) of fermentation (pH values 4.5). Results are expressed as means±SD; values are means of duplicates from three separate runs. Means with different lowercase superscript letters are significantly different (p<0.05). N.D. – not determined.

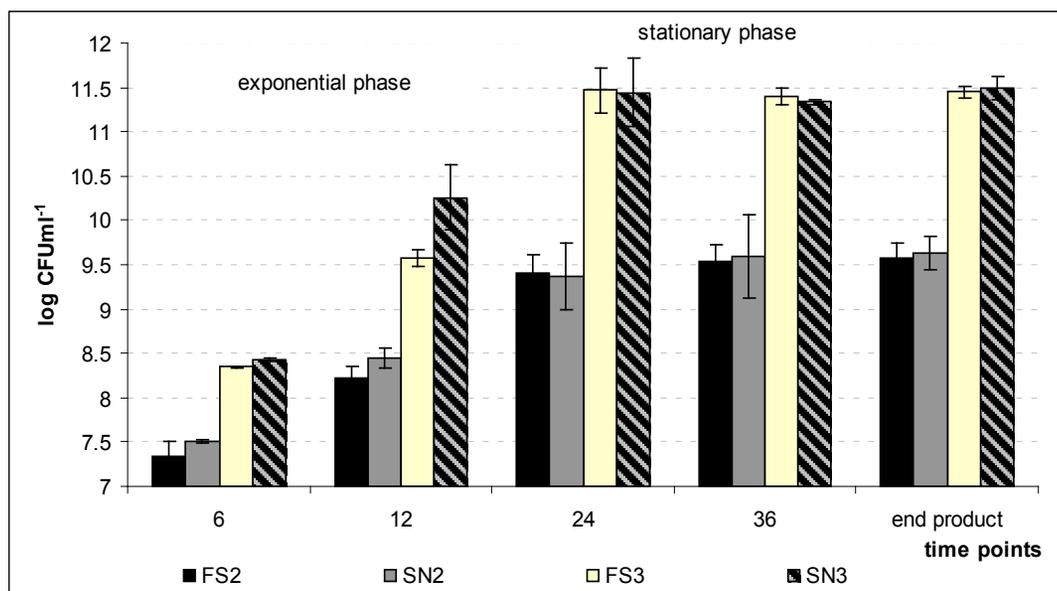


Fig. 2. Growth rate of *L. casei*-01 in soymilk supplemented with Synergy 1 vs. FOS, during fermentation at 37 °C. (Viable cell counts, log<sub>10</sub> cfu ml<sup>-1</sup>; time points, hours).

5.80±0.58 (app. 15%), while in low inoculum supplemented samples, non-significant decrease in pH was observed, 6.74±0.04 (≈ 1 %), compared to the initial value (Fig. 1). Regarding the prebiotic used, significant difference (p<0.05) was observed between the low inoculum samples (SN vs. FS) after 12 h of fermentation, when pH decline in Synergy 1 supplemented samples showed steeper and longer slope. For example, the pH value of the SN1 samples after 12 h of fermentation was 5.92±0.01 vs. 6.15±0.02 in the FS1 samples. Consequently, throughout the fermentation, pH values in Synergy 1 supplemented samples were significantly lower than in the FOS enriched samples. Few hours before termination of the fermentation (pH 4.5), the sharper decline (slope) of pH occurred in FOS samples, with the end pH value of 4.5 occurring app. 60 min faster than in Synergy 1 enriched samples. A similar decline pattern, with higher rate, was observed for high inoculated samples, probably because of the higher initial biomass of the probiotic (Fig. 1).

The viable cell counts of *L. casei*-01 in all end-products are summarized in Table 1.

At this stage, terminated fermentation (pH 4.5), significant difference in cell counts (p<0.05) was observed between the batches with low (0.005 and 0.01% w/v) and high inoculum (0.075, 0.1 and 1 % w/v) rates. Obtained values within batches were similar regardless the prebiotics used. As shown in Table 1, in high inoculum batches, the viable cell counts were significantly higher (p<0.05) in both SN and FS samples. Exceptions were the samples inoculated with 1% w/v *L. casei*-01, where the cell count was lower for 1 log in comparison with the other high inoculum rate samples.

Therefore, cell growth during fermentation was followed in inoculum of 0.01% w/v, representing the low inoculum samples (SN2 and FS2), and 0.075% w/v, representing the high inoculum samples (SN3 and FS3). The cell counts were determined at 6, 12, 24 and 36 h and at the end of fermentation (Fig. 2). Time points were cho-

sen based on changes in pH values observed during fermentation.

The growth rate of *L. casei*-01 in SN vs. FS samples was significantly higher during the exponential (log) phase of growth. The exponential phase of *L. casei*-01 associated with FOS lasted few hours longer regardless of the inoculum rate. Despite the variations observed in log phase, there was no significant difference in viable cell counts of *L. casei*-01 associated with FOS vs. Synergy 1 in the stationary phase of growth and at the end of fermentation.

Growth rate and viability of probiotics in beverage depends on the strain used, the initial inoculation, incubation temperature, fermentation time, availability of nutrients, and growth promoters (prebiotics). The ability of microorganisms to utilize prebiotics is strain- and substrate-specific (Shah, 2001; Pan et al., 2009). As for the degradation mechanism of inulin-type fructans, it has been reported that *L. paracasei* spp *paracasei* degrades inulin type fructans with different degree of polymerization (DP) (Makras et al., 2005). Furthermore, Goh et al. (2007) reported that *L. paracasei* 1195 has an extracellular enzyme that is cell wall associated and responsible for the degradation of large fractions of FOS and inulin. The results of sugar degradation analysis published by Tsujikawa et al. (2013), clearly indicated that *L. paracasei* DSM 20020 was capable of degrading the highly polymerized inulin into fructose and sucrose, whereas *L. paracasei* JCM 8130<sup>T</sup> failed to do so.

*L. casei*-01 showed a considerable growth with both prebiotics. Still, variations due to different prebiotics and inoculum rates were observed. Based on literature data, we assume that the variations in changes of pH and viable *L. casei*-01 counts, shown during fermentation, are due to the differences in chain lengths of the prebiotics used. The utilization of inulin-type fructans is strain specific. Both prebiotics were inulin-type fructans, but with different DP. Namely, the DP of FOS varies from 2 to 60 with an average DP of >10, while the commercial Synergy 1 mixture is composed of oligofructose (DP 2-8; DP<sub>av</sub> = 4) and long-chain inulin fraction (DP 10-60; DP<sub>av</sub> = 25), known as HP inulin (Roberfroid, 2007).

Our results correspond to those published by Perrin et al. (2002), who showed that shorter chains were the first to be consumed by probiotic bacteria. Furthermore, Aryana et al. (2007) reported lower pH in a medium with *L. casei* and short chain length oligofructose P95 (DP<sub>av</sub> = 5), compared to long and medium chain inulins. Therefore, the faster growth rate during exponential phase of growth in SN samples compared to FS samples was due to better utilization of short chain oligofructose from Synergy 1 mixture. This was followed by utilization of inulin, but with slower rate than FOS, which resulted in longer fermentation time to determined pH of app. 4.5 in SN samples. However, there was no significant difference in cell counts between the end-products with Synergy 1 and FOS, with the corresponding inoculums.

### *Changes of pH and viability of L. casei-01 during storage of prebiotic fortified soymilk*

The acidity and cell viability counts were determined after storage at 4 °C for two and four weeks. The pH values declined insignificantly with time, while for cell counts a significant change was observed, depending on the prebiotic used. Namely, during two weeks of storage at 4 °C, the cell counts in SN samples were stable. However, after 4 weeks, they significantly decreased. For example, in samples with inoculum rate of 0.01% w/v (SN2) and 0.075% w/v (SN3), they decreased to 9.10±0.14 and 10.45±0.36 log<sub>10</sub> cfu mL<sup>-1</sup>, respectively. The results are in agreement with the results obtained by Donkor et al. (2008), who reported a general decline in viable cell counts during storage at 4 °C from 21 to 28 days for *L. acidophilus* LAFTI L10, *B. lactis* LAFTI B94 and *L. casei* LAFTI L26. The viable cell counts of *L. casei*-01 in FS samples declined significantly after 2 weeks of storage. Namely, in the end-product with the inoculum rate of 0.075% w/v (FS3), the viable cells decreased from 11.45±0.06 log<sub>10</sub> cfu mL<sup>-1</sup> to 9.44±0.06 log<sub>10</sub> cfu mL<sup>-1</sup>.

Nevertheless, the obtained values were above the recommended minimum for probiotic beverages, which characterize the product as functional food. This is in agreement with studies reporting that high chain prebiotics maintain the viability of probiotics at level above the recommended minimum, up to 4 weeks of storage at 4°C (Aryana et al., 2007).

### *Peptide profile*

Probiotics, including *L. casei*-01, have a complex proteolytic system that enables them to hydrolyze soy proteins and grow in soymilk (Donkor et al., 2007). During hydrolysis the peptides with various biological effects are produced (Gibbs et al., 2004)

Protein patterns in fermented soy products have raised an increased interest because of their health benefit effects, especially in cardiovascular diseases since soy proteins were approved by the Food and Drug Administration in 1999 (FDA 1999). But, not all probiotic/synbiotic soy products are equal. Some variations are based on the probiotics and/or prebiotics used (Erdman et al., 2013). Results, obtained by SDS-PAGE analysis of soymilk protein profile during fermentation, confirmed the proteolytic activity of *L. casei*-01 as well as the influence of associated prebiotics, Synergy 1 and FOS, on the proteolytic rate (Fig. 3).

Approximately 90% of soy proteins are constituted of two major globulins, β-conglycinin (7S globulin α', α, β subunits; Mw ≥ 44 kDa) and glycinin (11S globulin acidic and basic subunits; Mw < 40 kDa) (Gianazza et al., 2003). SDS-PAGE protein profile of synbiotic soymilk demonstrated that these proteins were gradually degraded during fermentation (Fig. 3). At Lane 2 (FS3 sample, 12 hours of fermentation), the Mw of observed polypeptide bands were 14 kDa and 20 kDa (corresponding to Mw of glycinin basic

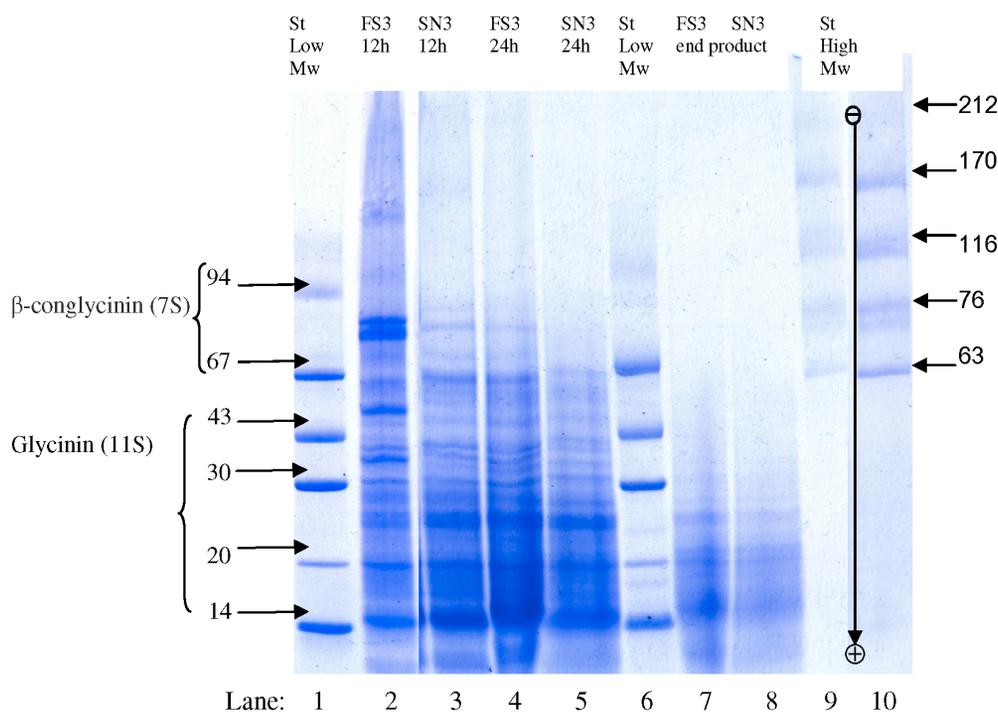


Fig. 3. SDS-PAGE electrophoretic profiles of proteins in synbiotic soymilk during fermentation: Lane 1 and 6=Low Mw Standard; Lane 9, 10, = High Mw Standard; Lane 2 = FN3, time point 12 h; Lane 3 = SN3, time point 12 h; Lane 4 = FS3, time point 24 h; Lane 5 = SN3, time point 24 h; Lane 7 = FS3 end-product; Lane 8 = SN3 end-product.

subunit), 28-30 kDa and 38-40 kDa (corresponding to Mw of glycinin acidic subunits) and 48-76 kDa (corresponding to Mw of  $\beta$ -conglycinin). At Lane 3 (SN3 sample, 12 h of fermentation), dominant bands were with Mw corresponding to glycinin subunits. The bands of  $\beta$ -conglycinin were less intensive, but still existed. Lane 4 (FS3 sample, 24 hours of fermentation) resembled Lane 3. Lane 5 (SN3 sample, 24 h of fermentation) demonstrated complete degradation of  $\alpha'$ ,  $\alpha$ , subunits of  $\beta$ -conglycinin.

As shown in Lane 7 (FS3 sample, end-product) and Lane 8 (SN3 sample, end-product), the  $\alpha'$ ,  $\alpha$ ,  $\beta$  subunits of protein  $\beta$ -conglycinin (7S) and the acidic chains of glycinin (11S globulin) were completely hydrolyzed. Observed polypeptide bands were with lower Mw (25, 20 and 14 kDa). The bands of basic glycinin of the end-products were less intensive compared to bands of samples obtained at 12 and 24 h of fermentation, indicating incomplete degradation. The bands in Lane 8 were less intensive than in Lane 7 indicating higher proteolysis.

As a consequence of delay in the growth rate of probiotic and pH drop, there was postponed soy proteins degradation in FS-enriched samples of soymilk.

#### Proteolytic activity

As it was confirmed by SDS-PAGE, there was an extended proteolysis of the soymilk proteins during fermentation. The result of protein hydrolysis is an increase in the

amount of free amino groups. The OPA-method, based on spectrophotometric detection of released  $\text{NH}_3$  groups upon hydrolysis, provided a direct measurement of the proteolytic activity. The extent of proteolysis in the end-products is shown in Table 1. The free amino acid content increased along with the enlargement of the inoculum rate. The values obtained for SN samples were significantly higher than those obtained for FS samples, indicating the higher proteolytic activity of *L. casei*-01 in association with the prebiotic Synergy 1. This was confirmed by the observation that the values for proteolysis in FS samples at inoculum rate of 0.075% w/v (FS3) were similar to those of inoculum rate of 0.01% w/v in SN samples (SN2).

The pH in SN samples was significantly lower than in FS samples throughout the fermentation, resulting in a significantly faster and higher hydrolysis and amount of generated free amino groups in the end-products. Higher proteolysis in SN samples is consistent with the findings of Ng et al. (2008) who claimed that the proteolytic activity of probiotics is growth-associated. In addition, De Giori et al. (1985) reported that by decreasing pH of the growth medium, the proteolytic activity is increased, mainly due to the alteration of active structure and hydrogen-ion equilibrium at the active site of the proteolytic enzymes upon pH changes.

*Optimization of inoculum rate in synbiotic soymilk product*

Evaluating the results obtained for pH change, viable cell counts and proteolytic profile during fermentation of soymilk with *L. casei*-01 in association with FOS and Synergy 1, respectively, we observed delay in pH change, biomass yield and proteolysis in FS samples in function of time as well as lower proteolytic activity in FS end-products. Moreover, the results for pH change during fermentation and proteolytic activity of *L. casei*-01 with inoculum of 0.075% w/v in association with FOS (FS3) were similar with the results obtained for significantly lower inoculum size, 0.01% w/v, of *L. casei*-01 in association with Synergy 1 (SN4). In addition, although there was no difference in viable cell counts between FS and SN end-products significantly lower cell counts were observed in FS samples after 2 weeks of storage at 4 °C, while SN samples were stable, showing a significant decrease after 4 weeks storage at 4 °C. Based on these findings, Synergy 1 protects the viability of *L. casei*-01 more efficiently than FOS, being more favorable for formulation of functional soymilk beverage.

Therefore, to optimize the inoculum rate of *L. casei*-01 to ferment the soymilk fortified by Synergy 1, the proteolytic activity in function of time was also determined. The sample with inoculum rate of 0.005% w/v (SN1) was excluded due to the low proteolytic activity, as well as all FOS fortified samples (Fig. 4).

The decreased pH during fermentation correlated with the increased proteolytic activity (e.g.,  $r = -0.885$ ,  $p = 0.0007$  and  $-0.902$ ,  $p = 0.0009$ , for SN2 and SN3, respectively).

In order to further optimize the inoculum of *L. casei*-01 for soymilk fermentation fortified by Synergy 1, the production of organic acids (lactic and acetic acids) in function of time was followed in the same synbiotic samples (SN2, SN3, SN4 and SN5).

The results obtained by HPLC showed an increased production of organic acids during fermentation. The concentration of lactic acid was higher than that of acetic acid in all batches (Fig. 5). Similar results were reported by Donkor et al. (2005), who made soy yogurt by fermenting the commercial soymilk using *L. acidophilus*, *B. lactis* and *L. casei*.

Results have shown that the probiotic inoculum of 0.075% w/v used to ferment the soymilk produced lactic and acetic acid in higher quantity compared to the inoculum of 0.01% w/v. Using an inoculum of 0.1% w/v, the production of lactic acid was not significantly changed (Fig 5a), while the production of acetic acid was increased (Fig. 5b). Acetic acid is an undesirable end-product in fermented soymilk due to its vinegary flavor and unpleasant sensory properties; therefore, the high production of lactic acid over acetic acid is desirable (Donkor et al., 2007; Donkor and Shah, 2008).

This indicates that the inoculum of 0.075% w/v can be considered optimal for preparation of the fermented soymilk beverage.

*ACE-inhibitory activity*

Proteolytic activity is an important attribute for production of peptides that could act as ACE inhibitors. The

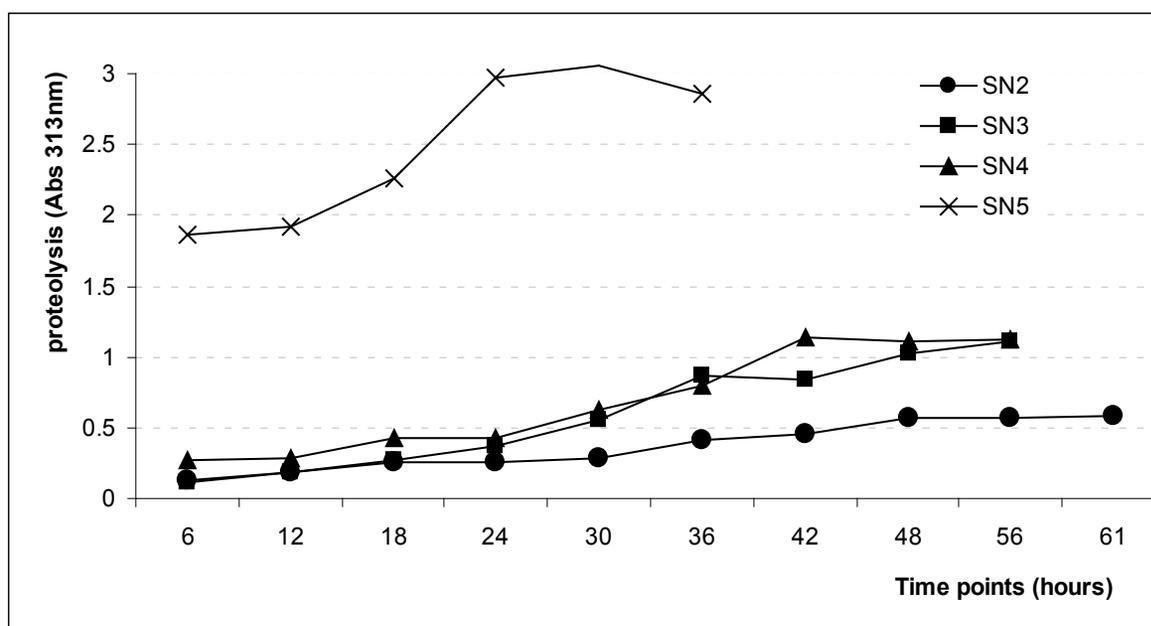


Figure 4. Absorbance vs. time curves for proteolysis during fermentation of Synergy 1-supplemented soymilk by *L. casei*-01 at different inoculum rates.

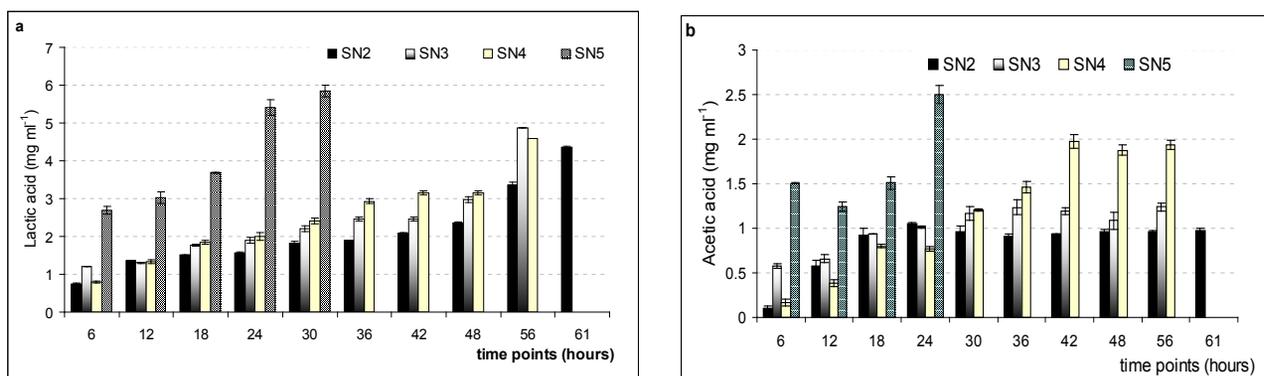


Fig. 5. Production of lactic acid (a) and acetic acid (b) in *L. casei*-01 and Synergy 1 fortified soymilk during fermentation with different inoculum rates.

development of fermented soymilk containing higher concentrations of released bioactive ACE inhibitors and viable probiotic may deliver health benefits of these functional compounds more efficiently (Donkor et al., 2005). For that purpose, the potential antihypertensive effect of the fermented soymilk beverage *in vitro*, based on percentage inhibition of the ACE activity was evaluated.

The %IACE of fermented Synergy 1-fortified end-products was evaluated in relation to different inoculums of *L. casei*-01 (SN1, SN2, SN3 and SN4 samples). Curves obtained by spectrophotometric measurements showed a nice linear decrease of absorbance. Sample without fermented soymilk showing 100% of ACE activity resulted in steeper slope than samples with fermented soymilk. Whole interval from 5 to 45 min was used for calculating the slope.

For the inoculum of 0.005 (SN1), 0.01 (SN2), 0.075 (SN3) and (SN4) 0.1% *w/v* *L. casei*-01, the %IACE was calculated as 71, 74, 77.8 and 78.4%, respectively (Fig. 6).

There was a significant increase in values obtained by increasing the inoculum from 0.005 to 0.075% *w/v*. Values

between high inoculum batches were similar. Namely, increasing the inoculum to 0.1% *w/v* the %IACE did not change, indicating the inoculum of 0.075% *w/v* optimal for fermented synbiotic soymilk with antihypertensive potential.

## Conclusion

This study showed that prebiotics FOS and Synergy 1 had different effect on the metabolic activity of *L. casei*-01 in soymilk. Both prebiotics provided high viability of *L. casei*-01 to above therapeutic minimum of 6 log cfu<sub>10</sub> ml<sup>-1</sup> after preparation and within 4 weeks of storage at 4 °C. But, faster cell growth during fermentation and higher viability during storage was observed in SN samples. Faster and higher proteolytic activity in samples fortified by Synergy 1 also indicated this prebiotic more favorable for preparation optimal formulation of functional soymilk beverage with *L. casei*-01 as probiotic. Protein profile of samples showed polypeptides with Mw lower than 25 kDa.

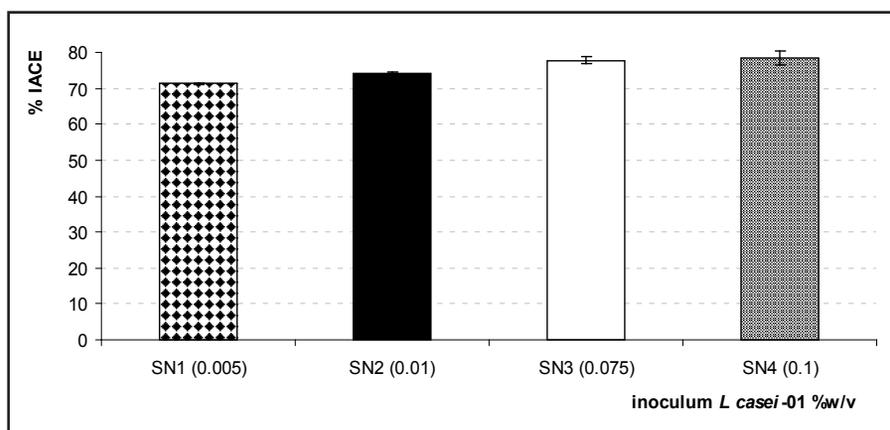


Fig. 6. %IACE in fermented 1.5% *w/v* Synergy 1-fortified soymilk end-products at different inoculum rate of *L. casei*-01.

The end products exerted appreciable *in vitro* IACE activity. Based on organic acid production and %IACE values in Synergy 1 fortified samples, the *L. casei*-01 inoculum rate of 0.01 for low dose and 0.075% w/v for high dose synbiotic beverage were chosen for further *in vivo* studies needed to assess the real antihypertensive potential of our synbiotic product. The development of fermented low-fat synbiotic soymilk with antihypertensive potential and high viable count of probiotic may deliver health benefits to target population with cardiovascular disease risk factors.

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## Резиме

# Синбиотски прехранбен производ базиран на соја – формулација и определување на антихипертензивниот потенцијал

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**Клучни зборови:** *L. casei*, олигофруктоза-збогатен инулин (Sinergy 1), фруктоолигосахарид (FOS), сојин напиток, АКЕ-инхибиција

Специфични цели на оваа студија беа да се избере пребиотик кој ќе обезбеди поголема виталност на пробиотикот *L. casei*-01 и соодветен инокулум на пробиотикот за подготовка на ферментиран напиток од соја со потенцијал да го инхибира ангиотензин-конвертирачкиот ензим (АКЕ). За овие цели беше подготвен напиток од сојино млеко збогатен со 1.5% м/в фруктоолигосахарид или олигофруктоза-збогатен инулин во кој беа додавани различни инокулуми на *L. casei*-01 (0.005-0.1% м/в). Метаболичката активност на пробиотикот беше следена преку рН, број на витални клетки, протеолитичка активност, создавање на органски киселини, додека терапевтскиот потенцијал на финалниот напиток преку инхибиција на АКЕ. Во текот на ферментацијата, виталноста на *L. casei*-01 го достигна препорачаното терапевтско ниво,  $9,58 \pm 0,035 \log \text{ cfu/mL}$  за примероците со низок инокулум (0,005 и 0,01% м/в) и  $11,54 \pm 0,13 \log \text{ cfu/mL}$  за примероците со висок инокулум (0,075 и 0,1% м/в), без разлика на користениот пребиотик. За време на ферментацијата, пониска рН, побрз клеточен раст и поизразена протеолитичка активност беше забележана со користењето на олигофруктоза-збогатен инулин што упатува на подобра искористливост на овој пребиотик

споредено со фруктоолигосахаридот. Хидролитичката активност беше зависна од користениот пребиотик, односно побрза со примената на олигофруктоза-збогатениот инулин, што беше потврдено со SDS-PAGE. Молекуларната маса на полипептидите, добиени со хидролиза на протеините, во синбиотскиот финален продукт беше пониска од 30kD. Инхибицијата на активноста на АКЕ изнесуваше 71, 74, 77 и 78% за инокулуми од 0,005, 0,01, 0,075 и 0,1% м/в, соодветно. Врз основа на овие резултати може да се заклучи дека комбинацијата на олигофруктоза-збогатен инулин (1.5% м/в) и ниско-дозен инокулум од 0,01% м/в или високо-дозен инокулум од 0,075% м/в на *L. casei*-01 е оптимална за подготовка на сојин напиток со задоволителен антихипертензивен потенцијал®.

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