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Comparative study on biochemical activity of the intestinal isolates *Lactobacillus* sp. V3 and *Bifidobacterium* sp. A71 in different substrates

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The two intestinal isolates *Lactobacillus* sp. V3 and *Bifidobacterium* sp. A71 were selected for soymilk fermentation according to their acidification activity in soymilk. Beetroot juice and carrot juice were chosen for soymilk supplementation as additional sources of carbohydrates and brewer's yeast as an extra source of nitrogen. The fermentation was carried out for eight hours at 42 ºC. The fermentation was monitored by standard analytical and microbiological tests for changes of acidity (decreasing pH and increasing acid content), the contents of soluble dry substances, sugars and α-amino acids as well as changes in the number of viable cells. The samples were collected at the beginning and subsequently every two hours until the end of the fermentation. The results showed that there were differences between the tested isolates in terms of their ability to ferment soymilk. The mix with brewer's yeast had a better stimulating effect on the growth of both strains compared to those with juices alone. In addition, the carrot juice stimulated the growth of *Bifidobacterium* sp. A71 better than beetroot juice, while the opposite effect was found for the growth of *Lactobacillus* sp. V3.

Keywords: *Lactobacillus*, *Bifidobacterium*, soymilk, fermentation, biochemical activity.

INTRODUCTION

During the last three decades, lactic acid bacteria have gained greatly in significance, since the acknowledgement of their positive effects on the health of humans.[1] Particular interest was focused on the *Lactobacillus* sp. and *Bifidobacterium* sp. strains, which exist as a part of the natural microflora, or those which inhabit the human intestines, the so called probiotics.[2] There are several ways in which probiotics contribute to the improvement of human health, although more importance is given to their share in the balance of the entire intestinal microflora. The production of harmful metabolites, procarcinogens and carcinogens in organisms is reduced by their antagonistic effect on a large number of pathogen and conditionally pathogen enterobacteria.[3]

One of the requirements for the emergence of benefitial effects is a sufficient number of viable cells in the fermented or non-fermented products. The growth of in-

testinal *Lactobacillus* sp. and *Bifidobacterium* sp. in cow's milk was shown to be inferior and prolonged (by approximately 20 hours) compared with standard yogurt starter cultures.[4,5] However, soymilk is known as a good medium for *Bifidobacterium* sp. strains.[6] In addition, soymilk also has a benefitial influence on health, since it contains many anticarcinogenic compounds.[7–9] The use of probiotic bacteria for fermentation can affect the nutritional quality and reduce the bad taste of soymilk as well.[10]

The goal of this work was to examine the influence of soymilk supplementation with additional sources of carbohydrates and nitrogen, on the growth rate and number of viable cells of the intestinal isolates *Lactobacillus* sp. V3 and *Bifidobacterium* sp. A71. From the economical point of view the effect of the supplements on the duration of the fermentation was also of interest.

EXPERIMENTAL

Soymilk preparation. The soymilk was prepared from soybean. The whole bean was soaked at a temperature of 60 ºC for two hours, and then ground in a blender for 10 min. The suspension was filtered through a manyfold gauze. The soymilk produced in such a manner had to be adjusted to a pH value of 6.5 and thereafter sterilized at 120 ºC for 15 min.

Additives preparation. The beetroot and carrot juices were obtained from fresh vegetables by squashing in a rotating juice maker, filtered and pasteurized at 70 ºC for 15 min.

The substrates based on soymilk were prepared as follows: soymilk with beetroot juice (5 % v/v), soymilk with carrot juice (5 % v/v), soymilk with mixed (1:1 w/w dry matter) beetrot juice and brewer's yeast autolysate (10 % v/v), and soymilk with mixed (1:1 w/w dry matter) carrot juice and brewer's yeast autolysate (10 % v/v).

The applied cultures. The cultures of *Lactobacillus* sp. V3 and *Bifidobacterium* sp. A71, utilized for the fermentation of soymilk, were isolated from the feces of a three year child and baby nourished at mother's breast, respectively. The isolation was performed by the decimal dilution method. After 72 hours of incubation at 37 ºC under microaerophilic conditions (Pro Gas - Torlak), the colony of *Lactobacillus* sp. V3 was recovered from a MRS agar plate (de Man, Rogosa, Sharp)[11] supplemented with 0.3 % bile (Torlak) and the colony of *Bifidobacterium* sp. A71 was recovered from a BMF agar plate (*Bifidobacterium* medium).[12] Both cultures were propagated in MRS broth supplemented with 0.5 % L-cystein (BDH)[13,14] and futher maintained in litmus milk at 4 ºC after incubation at 37 ºC for 24 h. The characterizations of the cultures were accomplished according to Bergey's Manual of Systematic Bacteriology[15,16] (morphological features of the cells, production of CO_2 from gluconate and ability to ferment 20 carbohydrates).

The preparation of inoculum. Single cultures of *Lactobacillus* sp. V3 and *Bifidobacterium* sp. A71 were propagated in MRS broth supplemented with 0.5 % L-cystein (BDH), and there from, the soymilk was sowed. After a threefold passage in soymilk, the 18-hour single cultures were prepared for the inoculums. The inoculums were diluted so that the initial number of viable cells in the substrates was 10^5 CFU (Colony Forming Units)/ml.

Determination of fermentation parameters. A quantity of 2 % (v/v) of inoculum of each culture was sowed into 150 ml of the utilized substrates. The fermentation progress was observed for eight hours at a temperature of 42 ºC, in order to determine the increasing H^+ concentration (pH-metric), increasing acid content (titrimetric) and decreasing soluble dry matter (refractometric). UV spectrophotometric methods were utilized for following the changes in the contents of carbohydrates and α-amino acids.[17] The rate of cells multiplication was determined by the standard decimal dilution method. The number of colony forming units were determined on MRS agar plates supplemented with 0.5 % L-cystein (BDH) after incubation at 37 ºC for 48 h under microaerophilic conditions (Pro Gas – Torlak). The results of the enumeration are expressed as log CFU/ml.[4,18]

The results of all the parameters are expressed as the average of three independent measurements.

RESULTS AND DISCUSSION

One of the main physiological differences between the genus *Bifidobacterium* sp. and *Lactobacillus* sp. is in the synthesis of acids during fermentation. *Lactobacillus* sp. strains produces only lactic acid, while the products of *Bifidobacterium* sp. strains are acetic acid and lactic acid in the molar ratio 1.5:1.[16] The synthesis of acids causes changes of the pH, the amount of soluble dry substances and the yield of acids in sub-

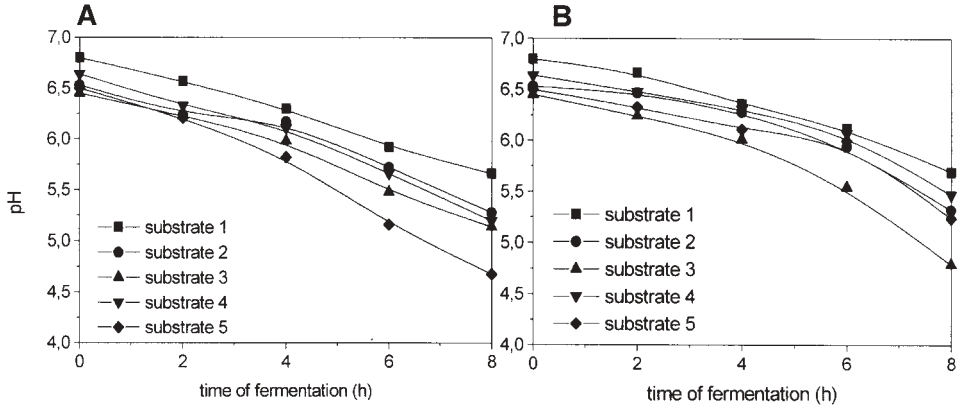

Fig. 1. The changes of pH during *Bifidobacterium* sp. A71 (A) and *Lactobacillus* sp. V3 (B) fermentation in different substrates; substrate 1–soymilk, substrate 2–soymilk with beetroot juice (5 % v/v), substrate 3–soymilk with mixed beetroot juice and brewer's yeast autolysate (10 % v/v), substrate 4–soymilk with carrot juice (5 % v/v), substrate 5–soymilk with mixed carrot juice and brewer's yeast autolysate (10 % v/v).

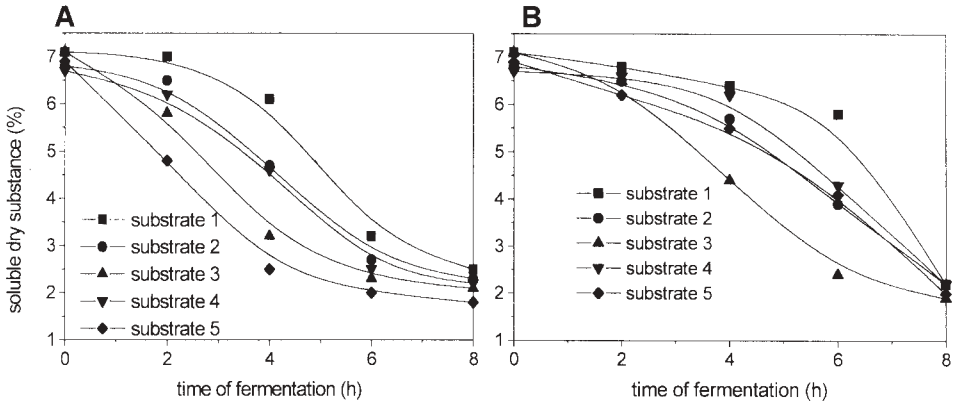

Fig. 2. The changes of soluble dry substance during *Bifidobacterium* sp. A71 (A) and *Lactobacillus* sp. V3 (B) fermentation in different substrates: substrate 1–soymilk, substrate 2–soymilk with beetroot juice (5 % v/v), substrate 3–soymilk with mixed beetroot juice and brewer's yeast autolysate (10 % v/v), substrate 4–soymilk with carrot juice (5 % v/v), substrate 5–soymilk with mixed carrot juice and brewer's yeast autolysate (10 % v/v).

strates such as soymilk. The results of the changes of these parameters for *Lactobacillus* sp. V3 and *Bifidobacterium* sp. A71 are shown in Figs. 1–3.

According to the results shown in Fig. 1 (A and B), the lowest value of the pH at the end of the fermentation time using *Bifidobacterium* sp. A71 was attained in substrate 5 (pH 4.58) where the point of soymilk clotting (pH 6) was reached after 4 h of fermentation as well as in substrate 3. In the other substrates fermented by *Bifidobacterium* sp. A71 clotting was achieved after 6 h of fermentation. On the contrary, the lowest pH at the end of fermentation was attained with *Lactobacillus* sp. V3 in substrate 3 (pH 4.79). The clotting pH value was reached after 4 h of fermentation. In the other substrates, clotting was achieved after 6 h of fermentation.

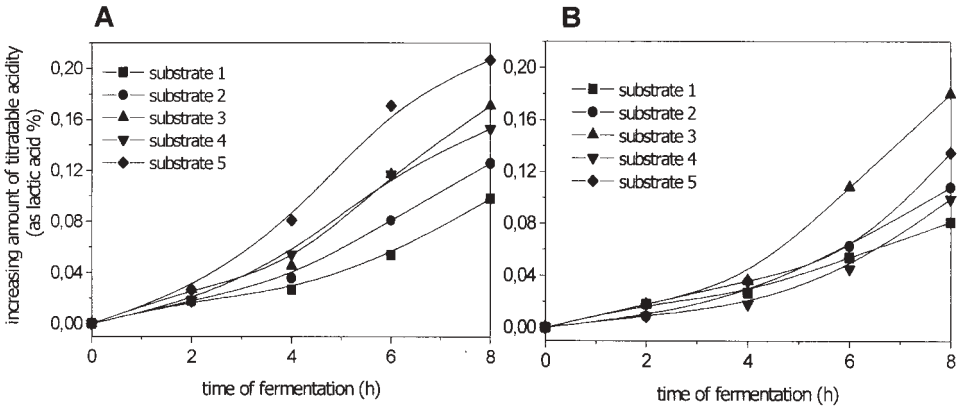

Fig. 3. The increasing amount of titratable acidity (as lactic acid, %) during *Bifidobacterium* sp. A71 (A) and *Lactobacillus* sp. V3 (B) fermentation in different substrates: substrate 1–soymilk, substrate 2–soymilk with beetroot juice (5 % v/v), substrate 3–soymilk with mixed beetroot juice and brewer's yeast autolysate (10 % v/v), substrate 4–soymilk with carrot juice (5 % v/v), substrate 5–soymilk with mixed carrot juice and brewer's yeast autolysate (10 % v/v).

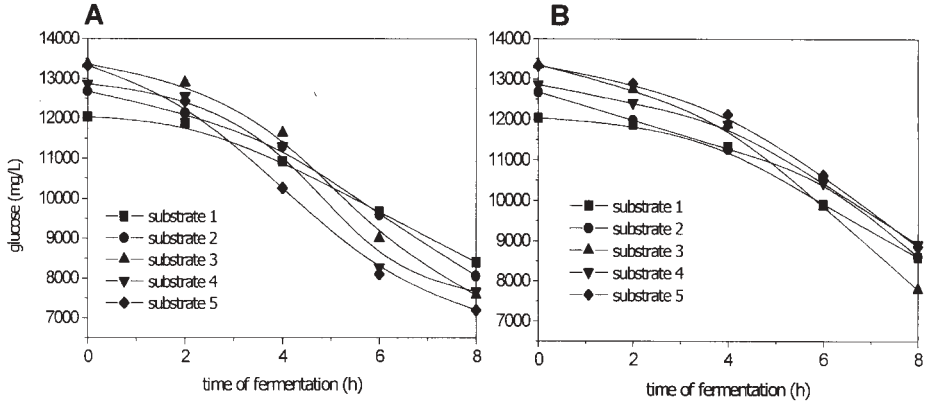

Fig. 4. The content of sugars (as glucose) during *Bifidobacterium* sp. A71 (A) and *Lactobacillus* sp. V3 (B) fermentation in different substrates: substrate 1–soymilk, substrate 2–soymilk with beetroot juice (5 % v/v), substrate 3–soymilk with mixed beetroot juice and brewer's yeast autolysate (10 % v/v), substrate 4–soymilk with carrot juice (5 % v/v), substrate 5–soymilk with mixed carrot juice and brewer's yeast autolysate (10 % v/v).

As a result of the pH decrease, the solubility of soybean proteins decreased too.[19] Clotting causes a reduction of the total solubility of the substances. So, the changes of the amount of soluble dry substrances can be one of the parameters indicating the activity in bacterial fermentations. According to the results in Fig. 2 (A and B), there is a difference in the rate of the decrease in the amounts of soluble dry substances in different substrates between the two strains. Activity of *Bifidobacterium* sp. A71 causes a more rapid decrease of the soluble dry substances present in all the substrates than the activity of *Lactobacillus* sp. V3. This difference is particularly expressed in substrates 3 and 5. However, the values of the amount of soluble dry substances in all the substrates at the end of the measurements are similar for both strains.

The total titratable acidity is expresed as the percent acidity increase in terms of lactic acid.[10] According to the results shown in Fig. 3 (A and B), a higher content of acids was attained with *Bifidobacterium* sp. A71, in all the substrates compared to the *Lactobacillus* sp. V3 strain. However, beetroot juice with brewer's yeast autolysate has a grater stumultive effect on acid production for *Lactobacillus* sp. V3 than for *Bifidobacterium* sp. A71. In contrast, carrot juice with brewer's yeast autolysate has a more intense effect on the synthesis of acids by *Bifidobacterium* sp. A71.

The acids originate from the transformation of sugars contained in the substrates. The content of sugars, expressed as glucose, decreases during fermentation. The results of these changes are shown in Fig. 4 (A and B).

Both strains cause a decrease in the content of sugars in the substrates during fermentation. The utilisation of sugars is more rapid with *Bifidobacterium* sp. A71 than with *Lactobacillus* sp. V3. The initial sugar content is higher in the substrates with brewer's yeast autolysate (3 and 5) compared to the other substrates as a consequence of the trehalose content from yeast. The beetroot and carrot juices increase the content of sugars in soymilk too. The highest total reduction of sugar content during fermentation with *Bifidobacterium* sp. A71 occurs in substrate 5 (6140 mg/L). With *Lactobacillus* sp. V3, the highest total reduction of sugars during fermentation occurs in substrate 3 (5559 mg/L).

Nitrogen is the main source for growth and the biosynthesis of the components of the bacteria cells. The available nitrogen in soymilk substrates comes from the amino acid residues in proteins and/or from free amino acids. The content of free amino acids decreases when they are present in amounts sufficient for the growth of cells. If the quantity of free amino acids is insufficient, the bacteria activate their proteolytic enzymes,[4] so that their content in the substrates might increase. The results of the changes of the α-amino acid content during fermentation are shown in Fig. 5.

As it emerges from Fig. 5 (A and B), the utilisation of α-amino acids show similar trends to that of the sugars. The highest reduction of α-amino acids during fermentation with *Bifidobacterium* sp. A71, occurs in substrate 5 (176.6 mg/L) and in substrate 3 (155.5 mg/L) with *Lactobacillus* sp. V3.

The fermentation rate is limited by the multiplication of the cells during the incubation time. The changes in the number of viable cells during fermentation of soymilk based substrates are shown in Fig. 6.

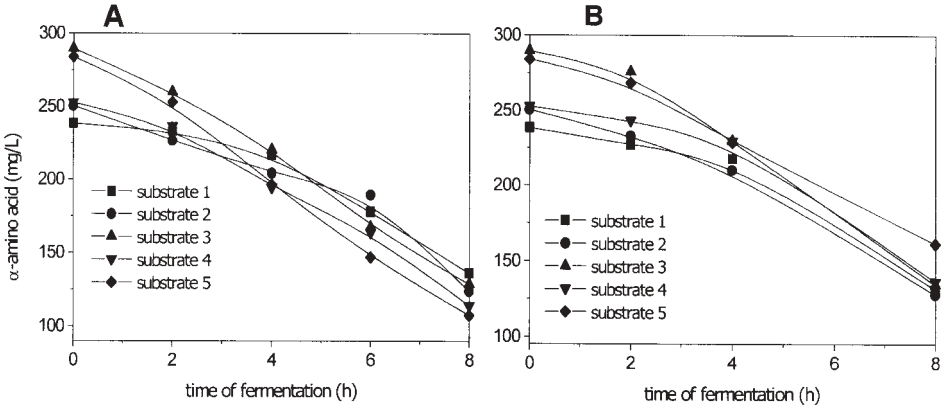

Fig. 5. The changes of the α-amino acid content during *Bifidobacterium* sp. A71 (A) and *Lactobacillus* sp. V3 (B) fermentation in different substrates: substrate 1–soymilk, substrate 2–soymilk with beetroot juice (5 % v/v), substrate 3–soymilk with mixed beetroot juice and brewer's yeast autolysate (10 % v/v), substrate 4–soymilk with carrot juice (5 % v/v), substrate 5–soymilk with mixed carrot juice and brewer's yeast autolysate (10 % v/v).

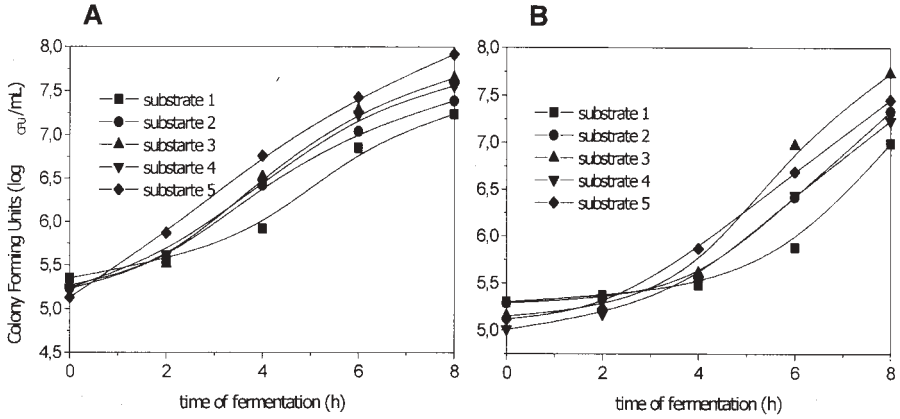

Fig. 6. The changes in the number of colony forming units (CFU) during *Bifidobacterium* sp. A71 (A) and *Lactobacillus* sp. V3 (B) fermentation in different substrates: substrate 1–soymilk, substrate 2–soymilk with beetroot juice (5 % v/v), substrate 3–soymilk with mixed beetroot juice and brewer's yeast autolysate (10 % v/v), substrate 4–soymilk with carrot juice (5 % v/v), substrate 5–soymilk with mixed carrot juice and brewer's yeast autolysate (10 % v/v).

According to the results shown in Fig. 6 (A and B), supplementation with beetroot (substrate 2) and carrot (substrate 4) juices lead to an increase of the number of viable cells of both strains compared to soymilk (substrate 1). However, the addition of brewer's yeast autolysate with carrot juice has the greatest stimulative effect on the multiplication of viable cells of *Bifidobacterium* sp. A71 (≈ 8 log CFU/mL) compared to all the other substrates. The highest increase in the number of viable cells of *Lactobacillus* sp. V3 (> 7.5 log CFU/mL) is attained in soymilk with brewer's yeast autolysate and beetroot juice.

It was noticed that there are some differences in the biochemical activity of the two natural isolates in used substrates which are expressed as different velocity of fer-

mentation and different stimulative effect of added juices. These differences might be caused by distinct nutritional necessities and abilities of substrate utilisation.

The decrease in the α-amino acid content and simultaneous increase in the number of viable cells in the substrates during fermentation suggest that the content of free amino acids was sufficient for the initial growth of the isolates. However, the addition of brewer's yeast autolysate contributed to an additional expansion of the CFU of both strains. A similar influence of yeast extract on the growth of bifidobacteria and lactobacilli was noticed in another study.[20]

Lactic acid bacteria (LAB) are adapted for growth in milk, whose main saccharide is lactose. With the help of β-galactosidase, most LAB split lactose into glucose and galactose which are easily fermented. In contrast to other LAB, the strains of *Bifidobacterium* sp. can utilise α-D-galactosyl oligosaccharides, because they possess α-galactosidase.[21] It is claimed that these oligosaccharides are prebiotics (selective stimulants for beneficial microflora) because they reach the colon (the lower part of intestinal tract) in unchanged form. Soymilk contains two such oligosaccharides - rafinose and stachiose. According to the latest studies, raffinose has an advantage compared to other prebiotics which is related to the fact of stimulation of the growth of bifidobacteria.[22] This might be the reason for the more prosperous growth of *Bifidobacterium* sp. A71 in the soymilk-based substrates compared to *Lactobacillus* sp. V3.

According to Gomez nad Malcata,[13] carrot juice extract stimulates the growth of *Bifidobacterium* sp. Rašić and Kurman[23] noticed that there are two factors which cause the stimulation of bifidobacteria growth by carrot extract. One is a precursor of coenzyme A - pantheine phosphate and the other is an unidentified, thermostable and water-soluble compound.

The reason why beetroot juice has a greater stimulative effect on the growth and activity of *Lactobacillus* sp. V3, compared to carrot juice could not be satisfactorily explained. If the acknowledged composition of the substances contained in these vegetables are compared, the greater folic and ascorbic acid contents in beetroot are immediately visible. According to the literature, the majority of lactobacilli requires folic acid as an essential growth factor, but bifidobacteria do not.[4,15,16] Shah[5] noticed that ascorbic acid stimulates the growth of *Lactobacillus acidophilus* strains as a reductive substance, but this is not the case with the growth of *Bifidobacterium* sp.

CONCLUSION

The two examined intestinal isolates may be of significance for soymilk fermentation, because of their prosperous growth and biochemical activity in this substrate. According to the examined parameters, the supplementation of soymilk with beetroot and/or carrot juice and especially with brewer's yeast autolysate affects the biochemical activity of *Lactobacillus* sp. V3 and *Bifidobacterium* sp. A71 strains. In addition, the supplementation of soymilk can increase the number of viable cells, as well as accelerate the fermentation. This is significant from an economic point of view, because of the shortened time of fermentation. The supplementation of soymilk brings benefits from

the nutritional point of view as well. All the cited foodstuffs have a beneficial effect on human health and such products can be classified as functional food.

И З В О Д

УПОРЕДНА ИСПИТИВАЊА БИОХЕМИЈСКЕ АКТИВНОСТИ ИНТЕСТИНАЛНИХ ИЗОЛАТА *Lactobacillus* sp. V3 И *Bifidobacterium* sp. A71 У РАЗЛИЧИТИМ СУПСТРАТИМА

СУЗАНА ДИМИТРИЈЕВИЋ-БРАНКОВИЋ и ЈОСИП БАРАС

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Интестинални изолати *Lactobacillus* sp. V3 и *Bifidobacterium* sp. A71 су изабрани за ферментацију сојиног млека према показаној ацидификационој активности у сојином млеку. Сок цвекле и мркве су изабрани за обогаћивање сојиног млека као додатни извор угљених хидрата а аутолизат пивског квасца као додатни извор азота. Ферментација је вршена у току осам сати на 42 °C и праћена је стандардним аналитичким и микробиолошким тестовима који су обухватали промену ацидитета (пад pH и повећање садржаја киселина), промену садржаја растворне суве супстанце, промену садржаја шећера (изражених преко глукозе) и α-амино азота, као и промену броја живих ћелија. Узорковање је вршено пре почетка ферментације и на свака два сата до краја праћеног периода ферментације. Резултати су показали да постоје разлике између тестираних изолата у погледу способности ферментације сојиног млека. Смеша појединачних сокова са аутолизатом пивског квасца је показала бољи стимулативни ефекат на раст и биохемијску активност оба соја, у поређењу са самим соковима. Додатно, сок мркве је бољи стимулант за раст соја *Bifidobacterium* sp. A71 у поређењу са соком цвекле, док је обрнути ефекат утврђен за раст *Lactobacillus* sp. V3.

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