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THE EVALUATION OF USEFULNESS OF POTENTIALLY PROBIOTIC *LACTOBACILLUS* STRAINS AS COMPONENTS OF INDUSTRIAL STARTER CULTURES

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Key words: probiotics, *Lactobacillus*, starter culture, prebiotics.

Abstract

The aim of the study was to investigate the possible use of nine *Lactobacillus* strains, previously isolated from infants faeces, as components of industrial starter cultures for yoghurt, soft white cheese and hard cheese production. There were no strong antagonistic interactions between isolates and commercial probiotics and yoghurt strains. None of isolates was able to grow at 3°C, seven grew at 7°C, all of them at 12°C and 37°C and only three at 45°C. In the culture media containing 10.4% NaCl at pH 6.5 growth of four isolates was detected at 12°C, whereas six strains grew at 37°C. Tested isolates did not hydrolyze casein and arginine and did not produce H_2S. Inulins (HD, IQ, TEX, HPX) and maltodextrins (low and medium dextrose equivalent) added to the culture media stimulated the growth of isolates. The properties of all isolates enable their application in soft white and hard cheeses and strains 4a, 4b and 14 in yoghurts.

OCENA PRZYDATNOŚCI POTENCJALNIE PROBIOTYCZNYCH PAŁECZEK Z RODZAJU *LACTOBACILLUS* JAKO SKŁADNIKÓW SZCZEPIONEK PRZEMYSŁOWYCH

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Słowa kluczowe: probiotyki, *Lactobacillus*, szczepionki przemysłowe, prebiotyki.

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Abstrakt

Celem pracy było określenie możliwości zastosowania dziewięciu szczepów z rodzaju *Lactobacillus*, wyizolowanych z kału niemowląt, jako komponentów szczepionek przemysłowych do produkcji jogurtów, serów twarogowych i dojrzewających. Nie odnotowano silnych antagonistycznych interakcji między izolatami, komercyjnymi szczepami probiotycznymi i jogurtowymi. Żaden spośród badanych izolatów nie wykazał zdolności do wzrostu w temperaturze 3°C, siedem było zdolnych do wzrostu w 7°C, wszystkie rosły w 12°C i 37°C, a tylko trzy – w 45°C. W podłożach zawierających 10.4% NaCl i o pH 6,5 odnotowano wzrost czterech szczepów w temperaturze 12°C i sześciu w 37°C. Badane izolaty nie wykazały zdolności do rozkładu kazeiny i argininy oraz produkcji H_2S. Inuliny (HD, IQ, TEX, HPX) i maltodekstryny (nisko- i średnioscukrzona) dodane do podłoży stymulowały wzrost izolatów. Właściwości wszystkich izolatów umożliwiają ich zastosowanie w serach twarogowych i dojrzewających, a szczepów 4a, 4b i 14 w jogurtach.

Introduction

Probiotics are defined as live micro-organisms which when consumed in adequate amounts confer a health benefit on the host (*Probiotics*... 2006). When selecting probiotics safety and functionality aspects, such as: survivability of strains during passage through the gastrointestinal tract, antagonistic activity against pathogens, as well as their technological properties, should be considered at the first place (MORELLI 2007). Before using a probiotic strain as a component of an industrial starter culture the interactions between probiotic strain and starter culture micro-organisms should also be investigated, because of the possibility of occurrence of the negative interaction which may lead to decrease of viability and activity of the micro-organisms (SAARELA et al. 2000).

Probiotics are used in many food products such as different types of cheese, yoghurts and milk beverages (BERGAMINI 2009, OLSON, ARYANA 2008, ONG et al. 2007). These products are characterized by different acidity and consistency. The pH value of fresh milk is about 6.5 to 6.8 and it decreases to about 4.0 to 4.5 in yoghurts, 4.5 to 4.6 in soft white cheeses and 5.2 to 5.4 in hard cheeses (LITWIŃCZUK et al. 2004). From the technological point of view salts play an important role in food products. Salt content in hard cheeses reaches 1.4 to 1.9%. Another parameter which affects the microbial ability to grow is water activity (a_w). Water activity of cheeses ranges from 0.70 to 0.99, for extra hard and soft white cheeses (cottage), respectively, whereas in semi hard cheeses a_w values are around 0.90 (LIU et al. 1998).

The recent trend in food industry is to apply probiotics together with prebiotics. Prebiotics are dietary components that are not digested in the small intestine and reach the colon intact. They influence the gastrointestinal microbiota, affect flavour and texture of products (CASTRO DE et al. 2009, GUGGISBERG et al. 2009, WANG 2009), but also stimulate the growth

of probiotics and increase their viability in food products (DONKOR et al. 2007). The knowledge of the ability of the probiotic micro-organisms to grow in the presence of prebiotics is crucial in composition of synbiotics products, in which pro- and prebiotics are used.

In the earlier studies (MODZELEWSKA et al. 2003) *Lactobacillus* strains were isolated from faeces of 24 infants. All isolated strains (20) exhibited strong antimicrobial activity against potentially pathogenic and technological harmful Gram-positive and Gram-negative bacteria. The characteristics of isolates, including chemotherapeutic resistance, survivability at low pH values and elevated concentration of bile salts, enabled selection of 9 strains with the biggest chances to survive in the gastrointestinal tract environment (MODZELEWSKA-KAPITUŁA et al. 2008). The aim of the present study was to select the isolates, which may be used as components of starter culture for yoghurt, white, soft and hard cheeses. Therefore, the interactions among isolates and some commercial probiotic and yoghurt strains, ability of isolates to grow at conditions present in production and storage of food products (pH value, temperature, NaCl content) and their proteolytic activity were investigated.

Materials and Methods

The test bacteria were *Lactobacillus* strains (*L. plantarum* strains marked as 1, 4b, 14, 18a, 20a, 20b, 22b, 24 and *L. fermentum* 4a). Before the experiments the strains were cultured in MRS broth (Merck, Darmstadt, Germany) at 37°C for 24 h.

The interactions between isolates and commercial strains with probiotic properties: *L. rhamnosus* 705, *L. casei* 163, *L. acidophilus* NCFM, *L. acidophilus* 74-2, *B. lactis* 420 (Danisco Biolacta, Olsztyn, Poland) and components of yoghurt starter culture: *L. delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus* (isolated from natural yoghurt, Bacoma SA, Warsaw, Poland) were studied using well diffusion method (MODZELEWSKA-KAPITUŁA, MARIN-INIESTA 2005). The type of mutual interactions (stimulation or inhibition) that isolates exhibited towards commercial cultures as well as that of commercial cultures towards isolates, were determined.

In order to study the influence of physical and chemical factors on growth of isolates MRS broth at different pH values: 6.5 (pH of fresh milk) and 5.0 (approximate pH of hard cheese) was used. The MRS broth contained 0.0, 3.3, 6.7 and 10.4% of NaCl, which corresponded to water activity 1.0, 0.98, 0.96 and 0.93, respectively (Thermoconstanter, Novasina AG, Zurich, Switzerland). Bacterial cultures (0.01 cm^3) were inoculated into media (9 cm^3) and incubated at different temperatures: 3°C (cooling temperature), 7 and 12°C (hard cheese

ripening temperatures), 37°C (optimal for bacteria isolated from human organism). The ability of isolates to grow at 45°C, that is used in yoghurt production, were also investigated in MRS broth (Merck). The growth of bacteria was controlled by absorbance measurement (spectrophotometer Heλios ε, Unicam Ltd. Cambridge, UK) at 650 nm with frequency adjusted to the rate of bacterial growth until two equal results were noted. If there were no differences in optical density of cultures the experiment was terminated after 7 days of incubation. In order to quantitatively determine bacterial population in the cultures the standard curve showing relationship between optical density and cells number was drawn.

The proteolytic properties of isolates were determined as their ability to casein and arginine hydrolysis and H_2S production. Hydrolysis of casein was tested on milk agar plates (BURBIANKA et al. 1983). Bacterial cultures containing 10^9 cfu cm^{-3} were inoculated in the form of stripes on surfaces of the plates and then incubated for 48 h at 37°C at anaerobic conditions (Anaerocult C, Merck). A strain was considered as possessing ability to hydrolyze casein if media around the stripe became transparent. Decomposition of arginine was tested in liquid medium containing arginine (BURBIANKA et al. 1983), which was inoculated with 0.01 cm^3 of bacterial cultures. After 24 h incubation at 37°C Nessler reagent was added to determine ammonia presence (ARICI et al. 2004). Ability of strains to produce hydrogen sulphide was tested on Triple Sugar Iron agar (TSI, Oxoid Ltd. Basingstoke, UK). During 14 days of incubation at 30°C the cultures were monitored daily for blackening the media indicating H_2S production (ARICI et al. 2004).

The isolates were tested for their ability to use the following prebiotics as a carbon source: inulin TEX (polymerization degree DP ≥ 9), HD (DP ≥ 9), IQ (DP = 9 to 12) (Sensus, Roosendaal, Netherlands), HPX (DP ≥ 23) (BENEO--Orafti, Tiennen, Belgium) and maltodextrins of different dextrose equivalent (DE): low (DE = 10.9) and medium (DE = 16.2) (Pepes SA, Łomża, Poland). Bullion Standard (Merck) was modified by addition of 0.5 to 2.5% of prebiotic. As controls Bullion Standard (Merck) and bullion with glucose were used. The bacterial cultures containing 10^4 cfu cm^{-3} were inoculated into media and incubated for 24 h at 37°C and then the counts of bacteria were determined on MRS agar (Merck) incubated for 48 h at 37°C at anaerobic conditions (Anaerocult C, Merck).

Results and Discussion

Commercial probiotic strains *L. acidophilus* NCFM, 74-2 and *B. lactis* 420 and yoghurt strains *L. delbrueckii sp. bulgaricus* and *S. thermophilus* did not

inhibit the growth of the strains isolated from infant faeces. It was noted that *L. rhamnosus* 705 slightly inhibited the growth of all isolated strains *L. plantarum* and *L. fermentum* (zone of inhibition 5 mm), whereas *L. casei* 163 slightly inhibited only *L. plantarum* 14 (zone of inhibition 5 mm). Isolates did not inhibit the growth of commercial strains except for *L. acidophilus* NCFM, which was slightly inhibited (zone of inhibition 4 mm) by *L. plantarum* 4b and 20a. The combination of strains which interact antagonistically should be avoided in the products in which a couple of probiotic strains or probiotics and starter culture are used (TIMMERMAN et al. 2004). This antagonistic interaction may be caused by acids, hydrogen peroxide and bacteriocins (which have an effect upon closely related bacteria) produced by lactic acid bacteria (TODOROV, DICKS 2005). The lack of strong antimicrobial interactions among tested isolates and commercial strains indicates the possibility of their collective usage in probiotic products.

None of the strains was able to grow at 3°C regardless of pH value of media and NaCl concentration (Table 1), thus during storage of food products in such low temperatures these bacteria will not proliferate. However, this do not disqualify them as a starter culture components as far as in the final product high enough number of probiotic cells will be provided. The isolates showed more diverse growth at 7°C. Strains: 4b, 14, 18a, 20b grew at 7°C at pH 6.5 and 5.0 and NaCl concentration up to 3.3%, whereas strains 4a and 20a did not grow under these conditions. Along with the increase of incubation temperature more intense growth of isolates was noted. All strains were able to grow at 12°C, pH 5.0 and a_w 0.96. Such conditions are prevailing during hard cheese ripening and ability of strains to grow under them indicate possible use of isolates in hard cheese production. The proliferation of probiotic bacteria during cheese ripening is highly desirable, because it enables to maintain high population in the final product. In media at pH value 6.5, incubated at 12°C growth of some strains: 1, 4a, 14, 20a was noted even when salt content reached 10.4%, which corresponded to a_w 0.93. In media at pH 6.5 incubated at 37°C eight strains were able to grow at the presence of 6.7% of NaCl, and only 4a did not. It was noted that strains 4a, 4b and 14 were able to grow at 45°C (Table 1), at which yoghurt fermentation traditionally proceeds. Thus, the strains can be used as adjunct cultures, with probiotic properties, without changes in technology parameters of yoghurt production.

Sodium chloride is a substance commonly used in the food industry, playing an important role e.g. in cheese ripening process (REINHEIMER et al. 1997). However it may decrease the growth and viability of bacterial cells (GOMES et al. 1998). PASSOS et al. (1993) and GÄNZLE et al. (1998) showed that higher than 3% addition of NaCl into media caused an inhibition of growth of lactic acid bacteria, whereas lower concentrations of salt (1 to 2%) positively affected

Table 1

The influence of temperature, pH and sodium chloride on the growth of potentially probiotic *Lactobacillus* strains

Parameter			Strains								
pH	°C	% NaCl	1	4a	4b	14	18a	20a	20b	22b	24
5.0	3	0–10.4	–	–	–	–	–	–	–	–	–
6.5	3	0–10.4	–	–	–	–	–	–	–	–	–
5.0	7	0–3.3	+	–	+	+	+	–	+	–	–
5.0	7	6.7–10.4	–	–	–	–	–	–	–	–	–
6.5	7	0	+	–	+	+	+	–	+	+	+
6.5	7	3.3	–	–	+	+	+	–	+	–	–
6.5	7	6.7–10.4	–	–	–	–	–	–	–	–	–
5.0	12	0	+++	+++	+++	+++	+++	+++	+++	+++	+++
5.0	12	3.3–6.7	++	++	++	++	++	++	++	++	++
5.0	12	10.4	–	–	–	–	–	–	–	–	–
6.5	12	0–3.3	+++	+++	+++	+++	+++	+++	+++	+++	+++
6.5	12	6.7–10.4	+	+	–	+	–	+	–	–	–
5.0	37	0	++	++	+++	+++	+++	++	+++	+++	++
5.0	37	3.3–6.7	+++	+++	+++	+++	+++	+++	+++	+++	+++
5.0	37	10.4	–	–	–	–	–	–	–	–	–
6.5	37	0–3.3	+++	+++	+++	+++	+++	+++	+++	+++	+++
6.5	37	6.7	+++	–	+++	+++	+++	+++	+++	+++	+++
6.5	37	10.4	+	–	+	++	++	–	+++	+	–
5.7	45	0	–	+++	+++	+++	–	–	–	–	–

+ 10^7 cfu cm^{-3}, ++ 10^8 cfu cm^{-3}, +++ ≥ 10^9 cfu cm^{-3}, – no growth

bacterial growth. The isolates tested in the present work showed very high tolerance to NaCl. It implies a possibility to use them in the production of hard cheeses which undergoes salting process.

It was noted that isolates showed diverse ability to grow at different temperature and NaCl concentration in spite of the common origin, which was gastrointestinal tract of infants. Thus it could be concluded that the ability to grow at different conditions is a strain, not only species, dependent property.

Tested strains grew similarly in media of pH 5.0 and 6.5, which correlates well with the results reported by MATAGARAS et al. (2003).

The isolates did not show proteolytic activity. None of them was able to hydrolyze casein and arginine and did not produce H_2S. Proteolytic properties of lactic acid bacteria which are used as components of starter or adjunct cultures in unripening fermented dairy products may negatively affect the sensory properties of products during many weeks of storage (KORNACKI et al. 1997). There are strains of lactic acid bacteria belonging to *L. fermentum* and *L. plantarum* species, which may be able to produce hydrogen sulphide (ARICI et al. 2004, LEE, SIMARD 1984). In hard cheeses H_2S produced during ripening contributes to desirable flavour profile (URBACH 1995). In other dairy products,

such as yoghurts and soft cheeses, the presence of H_2S indicates the decomposition of sulphur amino acids, and bacteria able to H_2S production are regarded as technologically harmful (ARICI et al. 2004). The fact, that tested isolates did not produce H_2S increased the possibility of their usage in fermented and non-fermented dairy beverages, and did not disqualify their application in hard cheeses production.

Application of synbiotics in production of fermented dairy products requires the determination of prebiotics influence on growth and activity of probiotic cultures. Growth of isolates in media containing prebiotics was better than in Bullion Standard and similar or frequently higher than in bullion with glucose (Table 2). After 24 h of incubation the counts of *Lactobacillus* sp. ranged from 10^7 to 10^8 cfu cm^{-3} and from 10^7 to 10^9 cfu cm^{-3}, in bullion with glucose and in prebiotic containing media, respectively. Prebiotics used in the study the strongest stimulated the growth of *L. plantarum* 4b, 14, 20a as compared to the growth of the isolates in media without glucose. The results obtained in the study show that lactic acid bacteria are characterized by diverse ability to use prebiotics as a carbon source. The similar findings were reported by BIELECKA et al. (2002) and Su et al. (2007).

Table 2

The growth of potentially probiotic *Lactobacillus* strains (log cfu cm^{-3}) after 24 h of incubation in media containing prebiotics

Medium	Prebiotic concentration	Strains								
		1	4a	4b	14	18a	20a	20b	22b	24
BS	0%	8.2	7.8	7.5	7.5	7.8	7.5	8.0	8.6	8.1
BG	0%	8.8	7.9	8.3	8.0	8.5	8.8	8.3	8.8	8.3
BS+IQ	0.5–2.5%	9.3–9.5	9.4–9.5	9.5– 9.9	9.8	9.4–9.7	9.3–10.0	9.4–9.6	9.5–9.8	9.4–9.7
BS+TEX	0.5–2.5%	8.4–9.0	9.3–9.5	9.8–9.9	9.8–10.0	9.4–9.6	9.3–10.0	9.7–9.8	9.3–9.5	9.4–9.9
BS+HPX	0.5–2.0%	9.2–9.3	8.8–9.0	8.7–9.2	9.4	9.3–9.5	9.3–9.4	8.3–9.4	9.4–9.6	9.5–9.7
BS+HD	0.5–2.5%	9.0–9.3	7.4–9.0	8.7–9.3	9.4–9.6	9.4–9.6	9.0–9.8	9.2–9.7	9.4–9.7	8.8–9.3
BS+m.m.	0.5–2.5%	8.7–9.0	8.7–8.8	9.1	9.4–9.8	9.2–9.7	9.3–9.6	9.3–9.8	9.3–9.4	9.1–9.4
BS+m.l.	0.5–2.5%	9.0–9.2	8.3–8.6	9.1	9.8–9.9	9.5–9.6	9.8–9.9	9.8–9.9	9.4–9.6	9.3–9.4

BS – Bullion Standard, BG – bullion with glucose, m.m. – maltodextrin of medium DE, m.l. – maltodextrin of low DE

Conclusions

1. There were no antagonistic interactions among yoghurt strains and *L. plantarum* and *L. fermentum* isolates, and amongst *B. lactis* 420 and isolates, thus they can be used together in yoghurt production.

2. Weak antagonistic interactions were noted among *L. rhamnosus* 705 and all of tested *L. plantarum* and *L. fermentum* strains. The antagonistic

interactions were also found between *L. casei* 163 and *L. plantarum* 14 as well as among *L. acidophilus* NCFM and *L. plantarum* 4b and 20. These interactions should be taken into consideration in composition of starter culture.

3. There is a possibility to use all tested isolates in soft and hard cheeses production because of their ability to proliferate in media of lowered water activity.

4. Strains *L. plantarum* 4b, 14 and *L. fermentum* 4a were able to grow at 45°C. Therefore they can be used in yoghurt production while maintaining the optimal for yoghurt bacteria temperature of fermentation.

5. All isolates grew well in the presence of prebiotics in sugar free media. These findings show the ability of isolates to use inulins: HD, IQ, TEX, HPX and maltodextrins of low and medium dextrose equivalent as a source of carbon.

6. Tested isolates did not show strong proteolytic properties, what enables their use in probiotic food production.

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