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ENZYME PROFILE OF LACTOBACILLI FROM TRADITIONAL BULGARIAN FERMENTED MILK PRODUCTS

Veronica Nemska[1], Svetla Danova[2], Nelly Georgieva[1]

[1] Department of Biotechnology, University of Chemical Technology and Metallurgy
8 Kliment Ohridski, 1756 Sofia, Bulgaria
E-mail: revn@abv.bg
[2] Department of General Microbiology, Stephan Angeloff Institute of Microbiology
Bulgarian Academy of Sciences, Sofia, Bulgaria

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ABSTRACT

In recent years, scientific community has focused the attention on the non-starter lactic acid microbiota of home-made dairy products as a source of new candidate-probiotic strains. Lactic acid bacteria (LAB) and in particular Lactobacillus spp. play a major role in acquiring the specific taste and aroma of the final product during the fermentation. For this purpose, 43 Lactobacillus strains, isolated from traditional Bulgarian katak, curd, yoghurt, white-brined and yellow cheese, were subjected to screening of their enzyme capacity. Twenty-five of the strains were pre-selected for determination of their enzyme profile by API ZYM system. It was established that most of the strains showed high leucine and valine arylamidase, β-galactosidase and α-glucosidase activity. Only a few strains showed N-acetyl-β-glucosaminidase, α-galactosidase, β-glucosidase and Naphtol-AS-BI-phospho-hydrolase activity. Alkaline phosphatase, trypsin, α-chymotrypsin, α-mannosidase, lipase (except L. lactis OC2), β-glucuronidase (except L. salicinus KC2) and α-fucosidase (except L. plantarum BS41 and L. lactis OC2) activity were not observed. In order to complete the obtained results, two qualitative tests for determination of the β-galactosidase and proteolytic activity of more than 30 Lactobacillus strains were also applied. Most of the investigated lactobacilli showed moderate proteolytic activity. According to their β-galactosidase activity, 34 % could be defined as active lactose fermenters, 23 % - as late lactose fermenters and 43 % - could not ferment lactose. The investigated Lactobacillus strains showed strain-specific enzyme capacity.

Keywords: *enzyme profile, β-galactosidase, lactobacilli, secondary microflora.*

INTRODUCTION

Fermented dairy products like katak, curd, yoghurt, white-brined and yellow cheese are a part of the Bulgarian national cuisine since ancient times. Their specific texture, taste and aroma are based on the characteristics of the raw material, natural microflora including lactic acid bacteria as well as the production technology.

The fermentation of traditional dairy products can be initiated by a natural, wild-type lactic acid microbiota that originates from the raw material or the environment and in the absence of starter culture added [1]. There is a recent trend exactly towards the isolation of such strains and their use as starter cultures in the fermentation due to their ability to preserve the uniqueness of the original product and the characteristics that make it popular. Non-starter lactic acid bacteria (LAB) are found to possess diverse enzyme activities which enrich the characteristic texture, taste and aroma of the end products [2]. When compared to starter cultures, they have also shown some differences in the growth rate and adaptation to particular substrates or raw materials, competitive behavior in mixed cultures and antimicrobial properties [3].

Lactobacilli are among the representatives of non-starter microbiota which are often reported in fermented dairy products [1]. They are of a particular interest due to their GRAS (Generally Recognized As Safe) status according to FDA and EFSA safety criteria and the recently increased number of *Lactobacillus* spp. with established probiotic properties. Lactobacilli are found

to restore homeostasis in intestinal disorders, synthesize various nutrients, reduce the blood cholesterol, boost the immune system, prevent the risk of certain diseases like cancer and intestinal inflammation, etc. [4, 5]. These positive qualities determine their wide application in the production of food and feed, probiotics, various chemicals, pharmaceuticals, etc. [6]. Due to the fact that the probiotic effects of lactobacilli are species- and strain-specific, a detailed investigation and selection of certain strains with desirable properties is needed [4].

In previous research, we focused on the isolation and identification of the lactic acid microflora, in particular *Lactobacillus* spp., from five traditional Bulgarian dairy products and the determination of the coagulation activity and organoleptic properties of some of these strains [7 - 9]. The aim of the present study was to evaluate the enzyme potential of 43 lactobacilli from the already formed laboratory strain collection and its role in the formation of the specific texture, flavor and aroma of these products in order to select those strains which are suitable for application in the food industry. Hence, the lactobacilli were subjected to a few modern, fast and reliable tests (like API ZYM system, ONPG discs) which allow the determination of their enzyme profile.

EXPERIMENTAL

Microorganisms, Media and Culture Conditions

Forty-three *Lactobacillus* strains from the collections of University of Chemical Technology and Metallurgy and the Stephan Angeloff Institute of Microbiology (Bulgarian Academy of Sciences, BAS) were preselected for the present study (Table 1). They were isolated from samples of home-made dairy products - katak, curd, yoghurt, white-brined and yellow cheese [10]. The pure

Table 1. Source and number of *Lactobacillus* isolates from traditional Bulgarian dairy products.

Fermented food	Raw material	LAB isolates
Katak	Sheep milk	*Lactobacillus* sp. S1 *Lactobacillus rhamnosus* S2 *Lactobacillus plantarum* S3 *Lactobacillus fermentum* S4
Curd	Goat milk	*Lactobacillus* sp. J6B
Yoghurt	Buffalo milk	*Lactobacillus plantarum* 1V, 2V, 3V, 7V, 8V, 10V *Lactobacillus hamsteri* 4V *Lactobacillus* sp. 5V, 6V *Lactobacillus fermentum* 9V
	Sheep milk	*Lactobacillus* sp. Ro32 *Lactobacillus rhamnosus* Ro33 *Lactobacillus delbrueckii* subsp. *bulgaricus* Ro34
	Cow milk	*Lactobacillus* sp. M1A
White-brined cheese	Sheep milk	*Lactobacillus plantarum* OC1, S5, S6, S8, S9, S12, Ko1 *Lactobacillus delbrueckii* subsp. *lactis* OC2
	Goat milk	*Lactobacillus plantarum* S7
	Buffalo and cow milk	*Lactobacillus plantarum* BS32, BS41 *Lactobacillus* sp. BS42 *Lactobacillus fermentum* BS31
	Cow milk	*Lactobacillus plantarum* S10 *Lactobacillus salivarius* subsp. *salicinus* KC2 *Lactobacillus* sp. H2A, H3D, H4D, KC1 *Lactobacillus fermentum* G7D
Yellow cheese	Cow milk	*Lactobacillus paracasei* S11
	Goat milk	*Lactobacillus plantarum* Kz1, Kz2, Kz3

cultures were stored at -20°C in de Man, Rogosa and Sharpe (MRS, HiMedia, India) broth, supplemented with 20 % glycerol. Before the assays, the strains were twice precultured under anaerobic conditions (Gas Pak 100 Anaerobic System, BD Bioscience, USA) in MRS broth, at 37°C for 24 h.

Enzyme Profile of LAB Strains

Enzyme profile of newly-isolated lactobacilli was determined by the API ZYM system, according to the instructions of the manufacturer (BioMerieux, France) [11]. Enzyme activity was estimated by a 5-point scale, based on the intensity of the staining - 0 – no change of color corresponds to a negative reaction, 5 – a maximum intensity of the staining, and values 3, 4 and 5 - being considered as a positive reaction.

β-Galactosidase Activity Assay

The β-Galactosidase activity of lactobacilli was determined with ONPG (o-nitrophenyl-β-D-galactopyranoside) discs, according to the instructions of the manufacturer (HiMedia, India) [12].

Proteolytic Activity Assay

The proteolytic activity of investigated *Lactobacillus* strains was determined according to a modified protocol of the agar-diffusion method [13], using Ca-caseinate agar (Fluka, Switzerland) and Milk agar (MPA medium, supplemented with 5 % sterile skimmed milk). The activity of exponential- and stationary-phase (24- and 72-hour) cultures of lactobacilli by the diameter of the obtained clear zones was reported.

RESULTS AND DISCUSSION

Assessment of enzyme activity is an important step in the determination of the technological relevance of LAB strains. Based on the obtained results, different strains can be selected for starter cultures and additives with beneficial effects on the quality of the final product. With this aim, 43 LAB strains were selected for further characterization [8].

In the present study, a semi-quantitative study of the enzymatic activities involved in the lipid, carbohydrate, protein and phosphate metabolism of 25 *Lactobacillus* strains was performed. The results obtained by using the 5-point scale of the API ZYM system were shown in Table 2.

Most of the lactobacilli showed high leucine and valine arylamidase and low or absent cystine arylamidase activity. In parallel, a lack of trypsin, α-chymotrypsin, esterase, esterase lipase and lipase activity (except strains *L. lactis* OC2, *Lactobacillus* sp. S1, *L. rhamnosus* S2, *L. plantarum* S3, *L. fermentum* S4, *L. paracasei* S11, *L. hamsteri* 4V) had to be pointed. The unbalanced concentrations of these enzymes can have a serious impact on cheese production. The ratio between proteinases and peptidases and their high levels may lead to disorders of cheese consistency and bitterness [14]. The presence of high peptidase and esterase-lipase and low lipolytic activity may also accelerate the maturation of cheese and has a beneficial effect on its flavor [15]. In addition, none of the strains showed alkaline phosphatase activity which is thought to be a common characteristic for LAB [2]. In terms of acid phosphatase, the activity was relatively low, except for *L. hamsteri* 4V and *L. plantarum* S6, S7 and S12. Among these strains, *L. hamsteri* 4V is the only one which had high acid phosphatase activity and may be applied in the degradation of phytate and the reduction of its anti-nutritional properties. Low phosphohydrolase activity was also observed for most of the strains, whereas for *L. rhamnosus* S2 and *L. plantarum* S5, S6 and S7 it was absent (Table 2).

According to the carbohydrate metabolism, almost all of the strains showed high β-galactosidase and α-glucosidase activity, whereas β-glucosidase activity was rather diverse. The high β-galactosidase activity presents the capacity of the lactobacilli to reduce the lactose intolerance and their ability to stimulate the growth of representatives of the genus *Bifidobacterium* in gastrointestinal tract (GIT). The high α-galactosidase activity was observed only for 6 strains, whereas the rest of them did not possess this activity. α-Galactosidase is an enzyme of a probiotic interest due to the fact that it is not synthesized by humans but has a great importance in carbohydrate utilization which makes it useful for obese or diabetic patients [16]. Most of the strains did not show to possess α-mannosidase and α-fucosidase activity (except *L. lactis* OC2 and *L. plantarum* BS41). In addition, high glucosidase and galactosidase activities and relatively low activity with respect to other carbon sources suggest that the strains under investigation may favor the utilization of galactooligosaccharides and glucooligosaccharides, which are widely used as prebiotic additives in the dairy industry.

Table 2. Enzyme profile of lactobacilli.

Legend: Enzymes: 1 – Alkaline phosphatase, 2 – Esterase (C4), 3 – Esterase Lipase (C8), 4 – Lipase (C14), 5 – Leucine arylamidase, 6 – Valine arylamidase, 7 – Cystine arylamidase, 8 – Trypsin, 9 – α-Chymotrypsin, 10 – Acid phosphatase, 11 – Naphthol-AS-BI-phosphohydrolase, 12 – α-galactosidase, 13 – β-galactosidase, 14 – β-glucuronidase, 15 – α-glucosidase, 16 – β-glucosidase, 17 – N-acetyl-β-glucosaminidase, 18 – α-mannosidase, 19 – α-fucosidase.

LAB strains	Enzymes																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
S1	0	2	1	0	5	4	1	0	0	1	1	5	5	0	3	1	0	0	0
S2	0	0	1	0	5	0	0	0	0	2	0	5	5	0	3	0	0	0	0
S3	0	2	1	0	4	3	0	0	0	2	2	4	4	0	3	1	2	0	0
S4	0	2	1	0	5	4	1	0	0	2	2	3	4	0	3	2	2	0	0
1V	0	0	0	0	4	2	0	0	0	1	1	0	3	0	3	4	3	0	0
2V	0	0	0	0	3	4	0	0	0	1	3	0	3	0	3	4	4	0	0
3V	0	0	0	0	3	3	1	0	0	1	2	0	3	0	3	4	2	0	0
4V	0	2	0	0	2	0	0	0	0	4	1	0	0	0	0	0	0	0	0
7V	0	0	0	0	4	4	2	0	0	2	3	0	3	0	3	4	3	0	0
8V	0	0	0	0	3	4	2	0	0	2	3	0	3	0	3	4	3	0	0
9V	0	0	0	0	4	3	0	0	0	1	2	0	2	0	2	2	1	0	0
10V	0	0	0	0	4	4	2	0	0	2	2	0	3	0	4	3	3	0	0
Ro34	0	0	0	0	3	0	0	0	0	1	1	5	5	0	0	0	0	0	0
S5	0	0	0	0	3	3	0	0	0	1	0	0	4	0	3	1	0	0	0
S6	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
S7	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0	0	0
S8	0	0	0	0	4	4	0	0	0	2	1	0	5	0	4	1	0	0	0
S9	0	0	0	0	4	4	1	0	0	2	2	0	5	0	4	1	0	0	0
S10	0	0	0	0	3	1	0	0	0	2	1	0	4	0	2	0	0	0	0
S12	0	0	0	0	3	3	0	0	0	0	1	0	3	0	0	1	0	0	0
KC2	0	0	0	0	3	2	1	0	0	2	2	0	3	1	3	2	2	0	0
OC2	0	2	1	1	2	1	1	0	0	2	1	0	2	0	3	4	3	0	1
BS41	0	0	0	0	2	2	0	0	0	1	1	0	2	0	2	3	2	0	2
G7D	0	0	0	0	5	0	0	0	0	1	1	0	4	0	0	0	0	0	0
S11	0	2	0	0	2	3	1	0	0	2	1	4	5	0	4	2	1	0	0

None of the lactobacilli possessed enzymes with a harmful effect like trypsin, α-chymotrypsin, β-glucuronidase and N-acetyl-β-glucosaminidase. Our results are in accordance with the investigations of González et al. [2] and Georgieva et al. [17]. The low or absent activity of these enzymes is promising from a probiotic point of view as they are involved in some pathogenic processes [2]. Among the investigated strains, only *L. salicinus* KC2 possessed low β-glucuronidase activity. It was established that LAB with such a low activity have the ability to synthesise antimicrobial substances or compete with other microorganisms for adhesion sites and nutrients in the GIT. They can inhibit the growth of some pathogenic or harmful bacteria like *E. coli* and *Clostridium* spp. [18]. Thus the tested lactobacilli may have therapeutic potential in the GIT when used as probiotic additives in dairy products. Kunduhoglu et al. [14] reported similar enzyme profiles during their investigation of microflora in Turkish Kargi tulum cheese.

Although the API ZYM system does only a semi-quantitative analysis, these results are important and allow the selection of LAB with useful technological and probiotic characteristics. The selected lactobacilli have a number of valuable enzyme activities, which makes them suitable starter cultures or additives in the production of products with various organoleptic properties.

In addition to the results, established by the API ZYM assay, a qualitative analysis of β-galactosidase activity of 30 *Lactobacillus* strains, isolated from 5 traditional Bulgarian lactic acid products - katak, curd, yoghurt, white-brined and yellow cheese, with commercial ONPG discs (HiMedia, India) was performed (Fig. 1). All tested strains were cultured in MRS broth for 24 h at 37°C under anaerobic conditions and the resulting exponential cultures were tested for β-galactosidase according to the manufacturer's instructions.

Summarised results clearly showed that after 3 h of cultivation, only 34 % of the strains could be identified as active lactose fermenters (they possess the enzymes β-galactosidase and permease) and 23 % of them are late lactose fermenters (they possess only β-galactosidase). Despite their dairy origin, 43 % of them could not ferment lactose. The highest β-galactosidase activity, commensurate with the control strain *E. coli* K12, was exhibited by 34 % of the strains (representatives of *L. plantarum* and *L. casei* group). *L. bulgaricus* Ro34 and *L. plantarum* S7 possessed lower activity but it was higher than that of 17 % of the strains with a pronounced low enzyme activity. The high β-galactosidase activity observed in the group of *L. plantarum* is also confirmed by investigations of other authors [19, 20]. At the same time, the high β-galactosidase activity of strains *L. rhamnosus* S2 and Ro33 isolated from katak and yoghurt is in contrary to the studies of Gheytanchi et al. [21]. When using the ONPG method, they reported low β-galactosidase levels (22.7 and 44.4 U/ml) of *L. rhamnosus* strains. Controversies are also found in the results obtained for β-galactosidase activity of *L. fermentum*. While *L. fermentum* G7D and BS31 showed a lack of enzyme activity, Palaniswamy and Govindaswamy [22] found the highest activity in 2 strains isolated from fermented millet porridge (kambu koozh).

β-galactosidase is an enzyme with an important industrial application as it plays an essential role in the reduction of some technological difficulties associated with the use of lactose in the food industry, relieves lactose intolerance [23], catalyses the galactoside synthesis reaction [24], etc. For this reason, the analyses for its determination are of great importance. Even though the ONPG test is only a qualitative method, it can serve as an initial point for further research. Additional biochemical characterization of strains with well-expressed

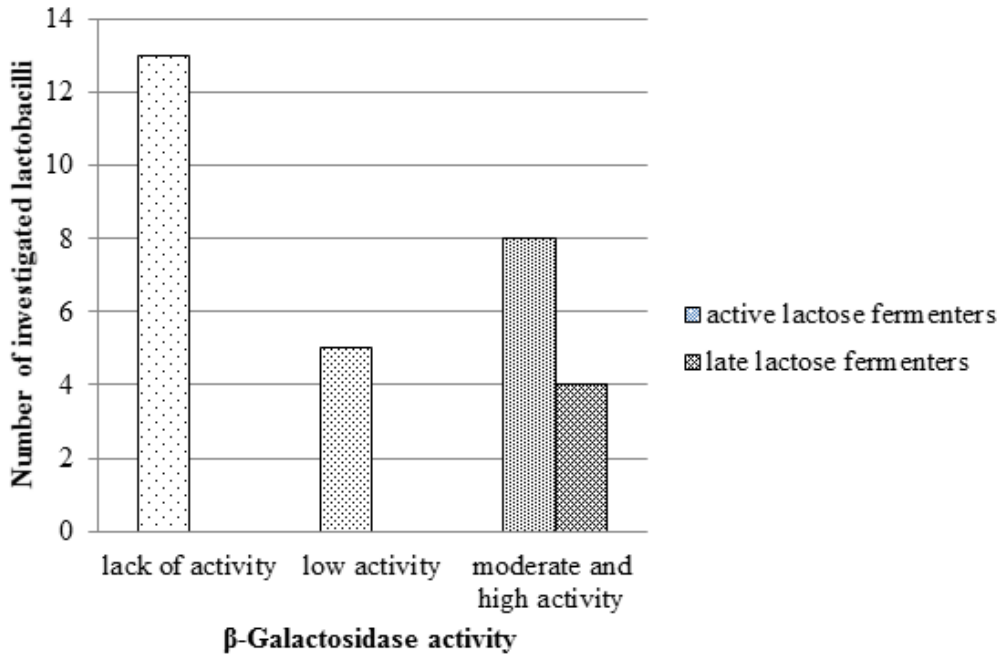

Fig. 1. β-Galactosidase activity of *Lactobacillus* strains.
Legend: Moderate and high activity: active lactose fermenters: *L. rhamnosus* S2, Ro33, *L. plantarum* S5, S6, S8, S10, S12, *L. paracasei* S11; late lactose fermenters: *L. bulgaricus* Ro34, *L. plantarum* S7, S9; Low activity: *Lactobacillus* sp. S1, J6B, *L. plantarum* BS41,Kz2, Kz3; Lack of activity: *L. plantarum* S3,OC1, BS32, Kz1, *L. fermentum* 9V, G7D, BS31, *L. lactis* OC2, *L. salivarius* KC2; *Lactobacillus* sp. H4D, H3D, BS42, KC1.

β-galactosidase activity should be performed, followed by quantitative analysis and laboratory in situ tests in a matrix imitating a real product.

The ability of lactobacilli to participate in the proteolysis of milk proteins is a species- and strain-specific characteristic and requires the study of the proteolytic activity of each newly-isolated strain. Our interest in this field was also provoked by the importance of the proteolytic system of LAB in cheese ripening, rapid growth in milk during fermentation, as well as the improved survival during storage [25]. In this regard, primary screening of the proteolytic activity and the ability of 34 *Lactobacillus* strains to hydrolyze casein has been performed. The analysis was carried out with a specially developed protocol for the agar-diffusion method, using two media: Ca-caseinate agar (Fluka, Switzerland) and Milk agar. During the cultivation of lactobacilli for 48 h at 37°C on Ca-caseinate agar, none of the strains formed a clear zone

Table 3. Total proteolytic activity of lactobacilli, isolated from traditional Bulgarian dairy products.

LAB strains	Clear zone in Milk agar, [mm]	
	24 h LAB cultures	72 h LAB cultures
Lactobacillus sp. S1	15	12.5
L. rhamnosus S2	16	15
L. plantarum S3	13.5	16
L. fermentum S4	14.5	14
Lactobacillus sp. J6B	13.5	15
L. plantarum 1V	12	15
L. plantarum 2V	13	14
L. plantarum 3V	13	14
L. hamsteri 4V	12.5	14
Lactobacillus sp. 5V	14	15
Lactobacillus sp. 6V	15	15
L. plantarum 7V	12	14
L. plantarum 8V	14	14
L. fermentum 9V	15	14.5
L. plantarum 10V	14	12.5
Lactobacillus sp. M1A	12	15
Lactobacillus sp. Ro32	14	15
L. bulgaricus Ro34	15	15
Lactobacillus sp.KC1	14.5	15
L. salivarius KC2	14.5	15
L. plantarum OC1	11.5	15.5
L. lactis OC2	13	13
L. fermentum BS31	15	17.5
L. plantarum BS32	14	16
L. plantarum BS41	13.5	14
Lactobacillus sp. BS42	15	17
L. fermentum G7D	15	15
L. plantarum Ko1	14	15
Lactobacillus sp. H2A	14	15
*Lactobacillus sp.*H3D	15	17
*Lactobacillus sp.*H4D	14	15.5
L. plantarum Kz1	15	16
L. plantarum Kz2	14	15.5
L. plantarum Kz3	14	15

around the wells, which required Milk Agar medium to perform the experiment. The results obtained for the total proteolytic activity of the studied *Lactobacillus* strains were presented in Table 3. All tested lactobacilli showed very clear zones on Milk agar media at the beginning of cultivation, whereas on Ca-caseinate agar no activity was observed. The lack of distinct clear zones on Ca-caseinate agar media is probably due to the weaker proteolytic and higher peptidase activity found in most of the strains, and also by the fact that the strains coagulated whole milk slower than skimmed milk [9].

The highest proteolytic activity on Milk agar media was observed in lactobacilli from yoghurt (*Lactobacillus* sp., 5V and 6V and *L. plantarum* 8V) and white-brined cheese (*L. plantarum* S12). According to the classification of Kunduhoglu et al. [14], they are defined as strains with moderate proteolytic activity (with a diameter of clear zone 13 - 20 mm). All other strains with a diameter of clear zone less than 13 mm are defined as strains with low proteolytic activity. The results on milk agar media also showed that 72-hour *Lactobacillus* cultures have better proteolytic activity than those in the exponential phase. It can be concluded that the proteolytic activity increases with time as a result of the initial cell lysis at the end of the fermentation. In addition, *L. plantarum* has shown to have a large number of genes encoding intracellular peptidases [26]. Donkor et al. [25] also found that the proteolytic activity of the LAB strains depends on the type of strain and duration of action. They observed a slight increase in the number of liberated amino groups and peptides from 0 to 12 h of fermentation in some strains, which then increased significantly in all strains (12 to 24 h). In contrast, however, Leclerc et al. [27] reported a linear increase in the number of free amino groups in *L. helveticus* at the end of fermentation.

The evaluation of the proteolytic activity of lactobacilli is essential for the selection of strains for application in cheese production. The lower proteolytic activity of the studied strains does not in any way limit their relevance and applicability. They play a significant role in secondary proteolysis due to their high peptidolytic potential, thereby increasing the number of short peptides and amino acids [28]. In comparison with starter cultures, the low proteolytic activity of probiotic bacteria has also been reported by other authors [29, 28], suggesting that the strains under investigation would find application as food supplements.

CONCLUSIONS

The technological properties of LAB are an important aspect in the selection of candidate-probiotic strains as they influence the quality and shelf-life of the final fermented products. The presence of enzymes with positive probiotic effect (like high aminopeptidase, β-galactosidase and α-glucosidase) and absence of those with harmful effect (like trypsin, α-chymotrypsin, β-glucuronidase and N-acetyl-β-glucosaminidase) makes the investigated lactobacilli suitable adjuncts for application in the food industry.

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