

Examination of some technological properties of lactic acid bacteria of the genera *Lactiplantibacillus* and *Levilactobacillus* isolated from spontaneously fermented sourdough. Part 1: Enzymatic profile

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Abstract. In the development of symbiotic sourdough starters, it is necessary to examine the enzymatic profile of all potential lactic acid bacteria (LAB) strains. The enzymatic profile of 3 strains of the genus *Lactiplantibacillus* and 2 strains of the genus *Levilactobacillus* was investigated using the API ZYM system (Biomerieux®, France) and in separate experiments the amylolytic and proteolytic activity were determined by the agar-diffusion method with wells. All *Lactiplantibacillus* strains possessed: leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase, phosphohydrolase, β -galactosidase, α -glucosidase, β -glucosidase and α -glucosaminidase. The two *Levilactobacillus brevis* strains possessed: lipase C4, esterase lipase C8, leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase. *Lactiplantibacillus plantarum* L1 demonstrated the highest amylolytic activity, and *Levilactobacillus brevis* X4 has the lowest. *Lactiplantibacillus plantarum* L1 exhibited the highest proteolytic activity, and *Levilactobacillus brevis* X4 - the lowest. The proteolysis was due to the production of inducible proteolytic enzymes by the LAB cells, as well as acid hydrolysis resulting from the lactic, acetic and other organic acids produced by the strains. The five LAB strains possess a rich and diverse enzyme profile, which is a prerequisite for their application in the development of symbiotic starters for sourdough bread.

1 Introduction

In bread biotechnologies, three main sources of enzymes are identified: endogenous enzymes present in the flour; enzymes related to the metabolic activity of the main microorganisms (yeasts and lactic acid bacteria); and exogenous enzymes that are purposefully added to the dough.

Endogenous enzymes contained in flour include amylases, proteases and lipases, which occur naturally in grains and play a key role in dough processing. These enzymes catalyze important biochemical reactions that affect the quality and texture of the final product [1, 2].

The metabolic activity of yeast and lactic acid bacteria, which are the main participants in the fermentation process, is also crucial for the dough development. Yeasts, mainly *Saccharomyces cerevisiae*, produce alcohol and carbon dioxide that help the dough rise and form its structure. Lactic acid bacteria produce organic acids that not only improve taste, but

also create a favorable environment for enzyme activity, regulating pH [3].

Industrial or exogenous enzymes that are added to the dough function as natural additives to optimize the production process and improve the qualities of bakery products [4]. In modern practice, innovations are introduced, including new enzymes with improved technological properties. The trend is to use complex mixtures of enzymes that act synergistically, increasing the individual effect on different flour components, such as gluten and starch [5]. The positive effects of exogenous enzymes include improvements in the consistency, firmness, aging and flavor of baked products [5]. These enzymes can increase the dough hydration, to improve the dough structure and increase the bread volume, which is especially important for industrial production.

The addition of sourdough affects the effectiveness of exogenous enzymes during fermentation, due to the lowering of the pH, which changes the enzyme activity

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and the metabolism of microorganisms. These enzymes interact with the metabolic activity of lactic acid bacteria in sourdough, releasing available nutrients and modifying other environmental factors, such as the production of organic acids and aromas [6].

Selection of the optimal combination of sourdough lactic acid bacteria and exogenous enzymes is essential for successful bread production in modern biotechnology. The development of specific cultures of lactic acid bacteria and their interaction with exogenous enzymes can lead to the improvement of the texture, taste and nutritional value of bakery products, which depends on consumer satisfaction and compliance with healthy trends [6].

Such an integrated approach in bread and bakery product biotechnology is fundamental to innovation in the food industry and to the adaptation of products to changing consumer demands. In this regard, the study of the enzyme profile of all newly isolated and identified strains of lactic acid bacteria is necessary, as it is involved in the selection of suitable strains for the development of starter cultures for bread sourdough.

The aim of the present work was to characterize the enzyme profile of newly isolated and identified strains of lactic acid bacteria, which are to be involved in the selection of suitable strains for the development of starter cultures for sourdough bread.

2 Materials and methods

2.1 Microorganisms

The examinations in the present study were carried out with the following strains of microorganisms: *Levilactobacillus brevis* Car, isolated from a spontaneously fermented cornmeal sourdough; *Levilactobacillus brevis* X4 isolated from spontaneously fermented sourdough from mortar flour; *Lactiplantibacillus paraplantarum* Ph3 and *Lactiplantibacillus paraplantarum* Ph5 isolated from spontaneously fermented wheat flour sourdough; and *Lactiplantibacillus plantarum* L1 isolated from spontaneously fermented spelt flour sourdough.

2.2 Examination of the enzyme activity profile

API ZYM system (BioMericux, France), according to the manufacturer's instructions.

2.3 Determination of the presence of amylolytic enzymes

15 cm³ of msLAPTg10-agar medium was poured into Petri dishes and in each of them, after solidification of the medium, 6 wells (d_{well}=6mm) were prepared with a corkscrew. Fresh 24-hour cultures of the samples were used. 60µL of the culture fluids of the strains were pipetted in the wells and three replicates were made for each sample. The result (the diameter of the clear zones in mm) is read at the 24th or 48th hour.

2.4 Determination of the presence of proteolytic enzymes

Sterile skimmed milk (10cm³ of skimmed milk for every 100 cm³ of sterilized melted LAPTg10-agar medium) was added to sterile melted LAPTg10-agar and 15 cm³ of the mixture were poured into Petri dishes. In each of them, after solidification of the medium, 6 wells were made with a corkscrew (d_{well} = 6 mm). Fresh cultures in the exponential growth phase (24-hour cultures) were used. 3 samples were prepared for each tested strain:

CL = culture fluid - 24-hour culture suspension of the strain

ASN = Acellular supernatant – obtained by centrifugation of the culture liquid and the obtained supernatant was transferred to a new tube.

CSPS = cell suspension in physiological solution - obtained by centrifugation of the culture fluid of the strain and washing the pellet once with physiological solution, followed by resuspension in physiological solution to the original volume of the sample.

Each sample was examined in triplicate, and the result (the diameter of the clear zones of casein hydrolysis in mm) was read at the 24th or at the 48th hour.

3 Results and discussion

3.1 Examination of the enzyme activity profile

According to Denkova, 2014, mesophilic homo- and heterofermentative lactic acid bacteria can be used in the development and production of bread sourdough. A number of scientific groups have found that bread with the best characteristics is obtained when using symbiotic sourdough starters containing both selected homo- and heterofermentative lactic acid bacteria. 1. Determination of the enzyme profile of *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus paraplantarum* Ph5, *Lactiplantibacillus plantarum* L1, *Levilactobacillus brevis* Car, *Levilactobacillus brevis* X4 [7].

The enzyme profile of *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus paraplantarum* Ph5, *Lactiplantibacillus plantarum* L1, *Levilactobacillus brevis* Car, *Levilactobacillus brevis* X4, isolated from spontaneously fermented sourdough, was investigated using the API ZYM kit system (Fig. 1., Fig. 2.). All *Lactiplantibacillus* strains possess the following enzymes: leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase, phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase and α-glucosaminidase, with some exceptions the enzyme activity values are comparable. *Lactiplantibacillus plantarum* Ph3 also possesses lipase C8, while *Lactiplantibacillus plantarum* Ph5 also possesses alkaline phosphatase (Fig. 1.).

Analogous to all representatives of the genus *Lactiplantibacillus* included in the present study, the strains *Lactobacillus plantarum* F3, *Lactobacillus plantarum* X2 and *Lactobacillus plantarum* LBRZ12 possess the same enzymatic activities, but *Lactobacillus plantarum* X2 and *Lactobacillus plantarum* LBRZ12

also possess lipase C8 and α -glucosaminidase, and *Lactobacillus plantarum* LBRZ12 exhibits alkaline

phosphatase, lipase C4, and lipase C14 activity as well [7].

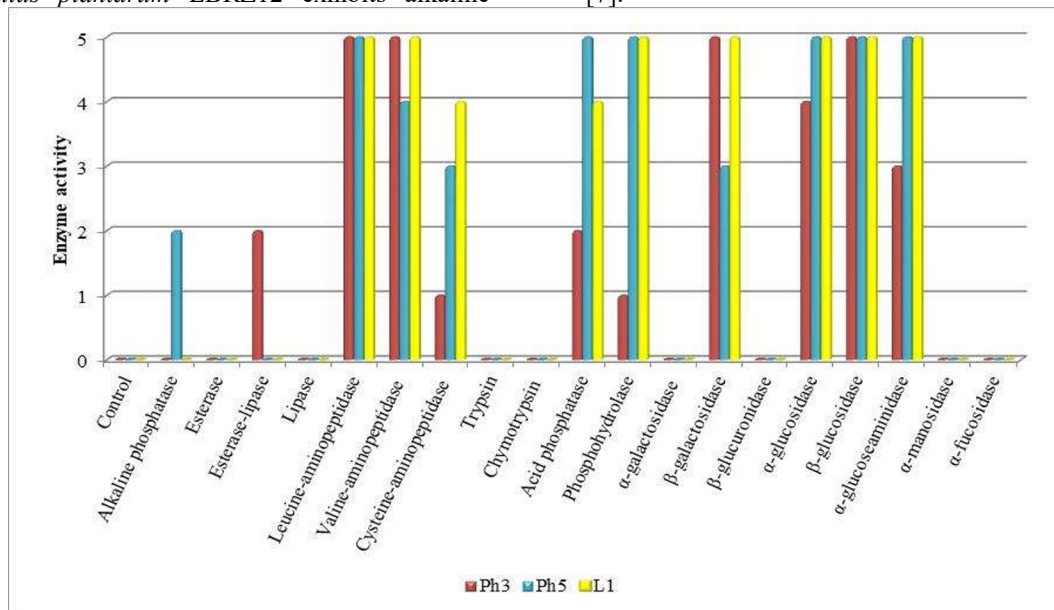


Fig. 1. Enzyme profile of *Lactiplantibacillus* strains - *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus paraplantarum* Ph5, *Lactiplantibacillus plantarum* L1. * Enzyme activity is determined on a color scale from 0 (absence of enzyme activity) to 5 (maximum enzyme activity)

Levilactobacillus brevis Car and *Levilactobacillus brevis* X4 possess the following enzymes: lipase C4, esterase lipase C8, leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolyase, α -galactosidase, β -

galactosidase, α -glucosidase, β -glucosidase, with some exceptions the values are comparable (Fig. 2.). *Levilactobacillus brevis* Car possesses both alkaline phosphatase and lipase C14 as well, while *Levilactobacillus brevis* X4 also exhibits α -glucosaminidase activity.

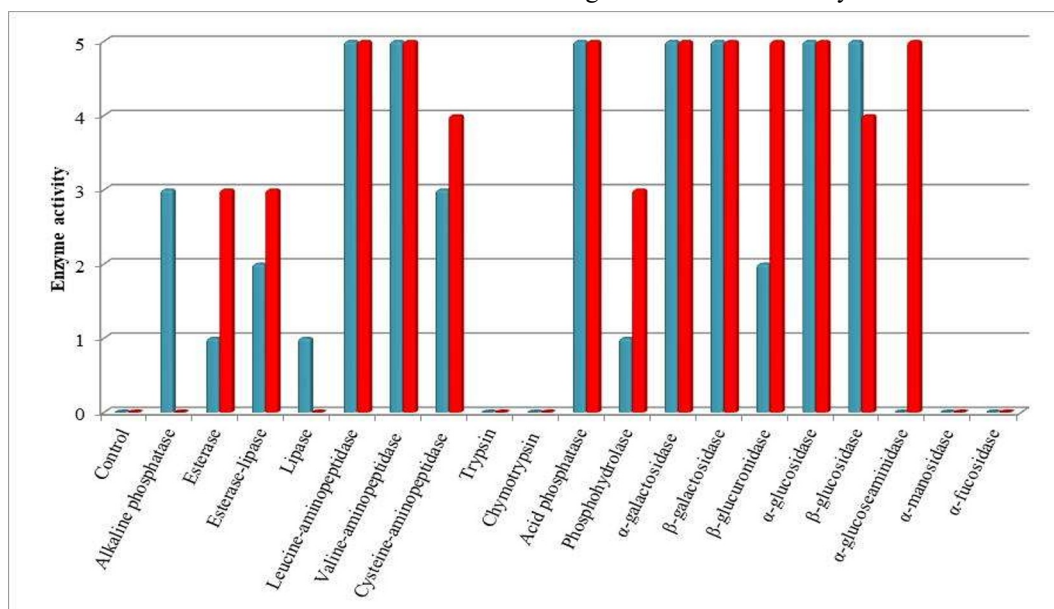


Fig. 2. Enzyme profile of *Levilactobacillus brevis* strains - *Levilactobacillus brevis* Car and *Levilactobacillus brevis* X4. * Enzyme activity is determined on a color scale from 0 (absence of enzyme activity) to 5 (maximum enzyme activity)

Analogous to *Levilactobacillus brevis* Car and *Levilactobacillus brevis* X4, *Lactobacillus brevis* LBRZ7 and *Lactobacillus brevis* LBRZ8 strains possess: esterase lipase C8, leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolyase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, with some exceptions the values are comparable. *Lactobacillus*

brevis LBRZ7 and *Lactobacillus brevis* LBRZ8 also exhibit alkaline phosphatase activity, and *Lactobacillus brevis* LBRZ7 possesses lipase C4 as well [7].

3.2 Determination of the amylolytic activity of *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus paraplantarum* Ph5, *Lactiplantibacillus plantarum* L1,

***Levilactobacillus brevis* Car, *Levilactobacillus brevis* X4**

The dough is characterized by a high content of starch, which is broken down by amylolytic enzymes. It is important to know the possibilities of lactobacilli to

break it down. In this regard, the amylolytic activity of the strains *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus paraplantarum* Ph5, *Lactiplantibacillus plantarum* L1, *Levilactobacillus brevis* Car, *Levilactobacillus brevis* X4 was investigated by the agar-diffusion method with wells (Table 1).

Table 1. Amylolytic activity of *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus paraplantarum* Ph5, *Lactiplantibacillus plantarum* L1, *Levilactobacillus brevis* Car, *Levilactobacillus brevis* X4. $d_{\text{well}} = 6\text{mm}$. The concentration of lactic acid bacteria was $10^{12} - 10^{13}$ CFU/cm³

Strain	Amylolytic activity
<i>Lactiplantibacillus paraplantarum</i> Ph3	11,17±0,24
<i>Lactiplantibacillus paraplantarum</i> Ph5	12,33±0,47
<i>Lactiplantibacillus plantarum</i> L1	15,67±0,47
<i>Levilactobacillus brevis</i> Car	13,67±0,47
<i>Levilactobacillus brevis</i> X4	10,33±0,47

The data from the triplicate experiments, reflected in Table 1, show that *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus paraplantarum* Ph5, *Lactiplantibacillus plantarum* L1, *Levilactobacillus brevis* Car, *Levilactobacillus brevis* X4 possessed high amylolytic activity, with the highest amylolytic activity being established in *Lactiplantibacillus plantarum* L1, followed by *Levilactobacillus brevis* Car, *Lactiplantibacillus plantarum* Ph5, *Lactiplantibacillus plantarum* Ph3, and with the lowest amylolytic activity being found in *Levilactobacillus brevis* X4 (Table 1). The representatives of *Levilactobacillus brevis* in the present study had slightly higher amylolytic activity than *Lactobacillus brevis* LBRZ7 and *Lactobacillus brevis* LBRZ8 isolated from spontaneously fermented vegetables [8]. The three representatives of the genus *Lactiplantibacillus* had slightly higher amylolytic activity compared to *Lactobacillus plantarum* LBRZ12, isolated from spontaneously fermented vegetables, and *Lactobacillus plantarum* X2 and *Lactobacillus plantarum* LBRZ12, isolated from spontaneously fermented sourdough [7].

3.3 Determination of the proteolytic activity of *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus paraplantarum* Ph5, *Lactiplantibacillus plantarum* L1, *Levilactobacillus brevis* Car, *Levilactobacillus brevis* X4

Dough proteins largely determine the structural-mechanical properties of bread. In this regard, the proteolytic activity of *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus paraplantarum* Ph5, *Lactiplantibacillus plantarum* L1, *Levilactobacillus brevis* Car, *Levilactobacillus brevis* X4 was investigated by the agar-diffusion method with wells. In parallel, the proteolytic activity of the culture fluid (CF), the acellular supernatant (ASN) and the cell suspension in physiological solution (CSPS) was determined (Table 2). *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus plantarum* L1, *Levilactobacillus brevis* Car were characterized by higher proteolytic activity, while *Lactiplantibacillus paraplantarum* Ph5 and *Levilactobacillus brevis* X4 had lower proteolytic

activity. The highest proteolytic activity was determined in *Lactiplantibacillus plantarum* L1, followed by *Levilactobacillus brevis* Car, *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus paraplantarum* Ph5, and the lowest proteolytic activity was established in *Levilactobacillus brevis* X4. Among the representatives of the genus *Lactiplantibacillus* with the highest proteolytic activity is *Lactiplantibacillus plantarum* L1, and among the representatives of *Levilactobacillus brevis* - *Levilactobacillus brevis* Car (Table 2). The obtained data on the diameters of the hydrolysis zones of the culture fluids (CF), acellular supernatants (ASN) and the cell suspensions in physiological solution (CSPS) show that the observed proteolysis was due to both the production of inducible proteolytic enzymes by the cells of the strains, some of which are associated with the cell wall and others are secreted into the environment, as well as acid hydrolysis resulting from the produced lactic acid, acetic acid and other organic acids. The two representatives of *Levilactobacillus brevis* had higher proteolytic activity compared to *Lactobacillus brevis* LBRZ7, *Lactobacillus brevis* LBRZ8 isolated from spontaneously fermented vegetables, and *Lactobacillus brevis* X1 isolated from spontaneously fermented sourdough [8]. The four representatives of the genus *Lactiplantibacillus* had higher proteolytic activity compared to *Lactobacillus plantarum* LBRZ12, isolated from spontaneously fermented vegetables, and *Lactobacillus plantarum* X2 and *Lactobacillus plantarum* LBRZ12, isolated from spontaneously fermented sourdough [7].

4 Conclusion

The two strains of *Levilactobacillus brevis* possess: lipase C4, lipase C8, leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase. *Lactiplantibacillus plantarum* L1 demonstrated the highest amylase activity and *Levilactobacillus brevis* X4 - the lowest. *Lactiplantibacillus plantarum* L1 showed the highest proteolytic activity and *Levilactobacillus brevis* X4 the lowest. Proteolysis was due to the production of inducible proteolytic enzymes by the lactic

acid bacteria cells, as well as to acid hydrolysis due to lactic acid, acetic acid and other organic acids produced by the strains. The five strains of lactic acid bacteria

possess a rich and diverse enzyme profile, which is a prerequisite for their application in the development of symbiotic starters for bread sourdough.

Table 2. Proteolytic activity of *Lactiplantibacillus paraplantarum* Ph3, *Lactiplantibacillus paraplantarum* Ph5, *Lactiplantibacillus plantarum* L1, *Levilactobacillus brevis* Car, *Levilactobacillus brevis* X4. $d_{\text{well}} = 6\text{mm}$. CF (culture fluid); ASN (acellular supernatant) and CSPS (cell suspension in physiological solution). The concentration of lactic acid bacteria was $10^{12} - 10^{13}$ CFU/cm³

Strain	Proteolytic activity	
	CF	ASN
<i>Lactiplantibacillus paraplantarum</i> Ph3	CF	20,33±0,47
	ASN	15,17±0,24
	CSPS	17,17±0,24
<i>Lactiplantibacillus paraplantarum</i> Ph5	CF	17,33±0,47
	ASN	13,67±0,47
	CSPS	13,17±0,24
<i>Lactiplantibacillus plantarum</i> L1	CF	25,17±0,24
	ASN	16,33±0,47
	CSPS	23,33±0,47
<i>Levilactobacillus brevis</i> Car	CF	21,67±0,47
	ASN	14,17±0,24
	CSPS	20,67±0,47
<i>Levilactobacillus brevis</i> X4	CF	14,67±0,47
	ASN	13,17±0,24
	CSPS	13,17±0,24

5 References

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