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Tsveteslava Ignatova-Ivanova¹
Maria Ananieva²
Radoslav Ivanov¹
Ilia Iliev³
Iskra Ivanova²

Biodiversity of lactic acid bacteria in Bulgarian wheat and rye flour

Authors' addresses:

¹Department of Biology, Shumen University,
9712 Shumen, Bulgaria.

²Department of General and Applied
Microbiology, Sofia University,
1164 Sofia, Bulgaria.

³Department of Biochemistry and
Microbiology, Faculty of Biology,
Plovdiv University,
24, Tsar Assen Str.,
4000 Plovdiv, Bulgaria.

Correspondence:

Tsveteslava Ignatova-Ivanova
Department of Biology, Shumen University,
115, Universitetska Str.,
9712 Shumen, Bulgaria
Tel.: +359 887160771
e-mail: radi_cvet@abv.bg

ABSTRACT

The microbial ecology of the flour fermentation is determined by ecological factors. Microbiological studies have revealed that more than 50 species of LAB, mostly species of the genus *Lactobacillus*, and more than 20 species of yeasts, especially species of the genera *Saccharomyces* and *Candida*, occur in this ecological niche. The flour microflora is composed of stable associations of lactobacilli and yeasts, in particular due to metabolic interactions. As shown for certain industrial flour processes, such microbial associations may endure for years, although the fermentation process runs under non-aseptic conditions. A reproducible and controlled composition and activity of the flour microflora is indispensable to achieve a constant quality of sourdough bread. Study on 4 natural flour types from various regions in Bulgaria – wheat flour from the villages of Okorsh, Dulovo and Melko, and rye flour from the village of Lilyak and the Archar mill. The isolates have been obtained through anaerobic cultivation on different media – MRS, mMRS 2% maltose, Elikor and mMRS 2% sucrose. The 10 isolates obtained have been analysed in terms of cell morphology, gas production on glucose, proteolytic activity and amylolytic activity. The species have been identified through molecular genetics methods, through 16S rRNA. The results indicate that the proteolytic and amylolytic activities are specific in terms of species and strains.

Key words: lactic acid bacteria, amylolytic activity, proteolytic activity, 16S rRNA

Introduction

Sourdough bread is a traditional product with great potential. This can only be achieved if the interactions between the lactic acid bacteria (LAB) and yeasts that populate the sourdough are understood (Gobbetti, 1998). Traditionally, sourdough has been used to produce many types of bread. Sourdough is a mixture of ground cereals (e.g., wheat or rye) and water that is spontaneously fermented. Sourdough fermentations improve dough properties, enhance both bread texture and bread flavor, and delay bread spoilage (Ehrmann & Vogel, 2005). LAB and

yeasts play a key role in sourdough fermentation processes (Hammes *et al.*, 1996; Gobbetti, 1998; De Vuyst & Neysens, 2005; Gobbetti *et al.*, 2005). Sourdough LAB have been intensively studied with respect to their carbohydrate metabolism (Gobbetti *et al.*, 1994a; Gänze *et al.*, 2007), proteolysis and amino acid metabolism (Gobbetti *et al.*, 1994b; Gänze *et al.*, 2007), lipid metabolism (Gänze *et al.*, 2007) and production of volatile compounds (Czerny & Schieberle, 2002; Hansen & Schieberle, 2005). Besides these general metabolic traits, specific metabolic properties have been recognized in sourdough LAB, such as the use of alternative electron acceptors, the production of antifungal

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compounds (Schnürer & Magnusson, 2005), the biosynthesis of exopolysaccharides (Tieking & Gänzle, 2005), and arginine catabolism (Rollán *et al.*, 2003). These metabolic traits of sourdough LAB highlight their adaptation to the sourdough environment. For instance, fructose-to-mannitol and arginine-to-ornithine conversion favor ATP generation and/or acid stress (Gänze *et al.*, 2007). Also, interactions between sourdough LAB and yeasts have been studied in detail (Gobbeti, 1998; De Vuyst & Neysens, 2005). The microbial growth and activity of LAB in sourdough are influenced by endogenous factors (e.g., flour carbohydrates and enzymes); process parameters (e.g., water content and temperature); and interactions between LAB, yeasts, and other bacteria (Gobbeti, 1998; Meroth *et al.*, 2003). Despite changes in raw material or the bakery environment, the sourdough microbiota is often remarkably stable, even for several years (Gänze *et al.*, 2003). The population dynamics of microbial food ecosystems have been studied mainly through microbiological analysis (Giraffa, 2004). In recent years, culture-independent methods have been developed to circumvent the limitations of conventional cultivation for the analysis of microbial communities in fermented foods (Ercolini, 2004). In this regard, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA fragments (16S rRNA PCR-DGGE) is frequently used as a relatively rapid and reliable cultivation-independent method to study the biodiversity and population dynamics of microbial communities (Ercolini, 2004; Van der Meulen *et al.*, 2007). A few studies have already described the use of PCR-DGGE to monitor the diversity and dynamics of LAB and yeast populations during sourdough fermentation processes (Meroth *et al.*, 2003; Randazzo *et al.*, 2005). Due to the large variety of cereals and fermentation conditions, the taxonomic composition of LAB microbiota found in sourdoughs worldwide is very diverse.

Materials and Methods

Strains

Ten strains of LAB, isolated from natural sourdoughs from different Bulgarian region were used in this study. The strains were cultured overnight (72 h) on Mann Rogosa Sharpe (MRS), Eliker, mMRS in supplement 2% sucrose and mMRS in supplement 2% maltose in pH 4.5 broth at 37°C and in limitation of oxygen.

Media

The strains were cultivated in MRS media with composition, per liter: glucose – 20; Tween 80 – 1; pepton from casein – 10.0; meat extract – 8.0; yeast extract – 4.0; K₂HPO₄ – 2.0; sodium acetate – 5.0; ammonium citrate – 2.0; MgSO₄·7H₂O – 0.2 and MnSO₄ – 0.05 g/l; media Eliker in composition (g/l): pepton from casein– 20.0; yeast extract – 5.0; gelatine – 2.5; glucose – 5.0; lactose – 5.0; sucrose – 5.0; askorbic acid – 0.5; sodium acetat – 1.5; sodium chloride – 4.0; mMRS in supplement 2% maltose and mMRS in supplement 2% sucrose. The pH of media MRS was adjusted to 6.5 with 1 N NaOH and media Eliker was adjusted to 7.0 with 1 N NaOH. The basic medias was sterilized by autoclaving at 121°C for 20 min, and carbohydrates supplemented were sterilized using 0.22 µm filters (Manisart®). An mMRS agar medium with different OS was prepared by adding 2% (w/v) sucrose and maltose to MRS agar.

Proteolytic activity assay on milk agar

Ability of Lactobacillus strains to hydrolyze casein on skin milk agar plates was tested as follows: Milk coagulation test was performed in 10% (w/v) reconstituted skin milk (RSM, Merck). Briefly, overnight cultures growing on MRS broth were harvested by centrifugation at 10.000 x g for 10 min at 4°C. After adjusting the pH at 6.5 by NaOH, the activity of the collected supernatants (100 µl) was determined on skin milk agar plates. The plates were incubated over night at 37°C. All experiments were performed in triplicate.

Amylolytic activity

Amylolytic activity assay was performed as previously described by the well diffusion method by using soft 0.8% agar. The cells of LAB cultivated in MRS broth were harvested by centrifugation at 10.000 x g for 10 min at 4°C. After adjusting the pH at 6.5 by NaOH, the activity of the collected supernatants (100 µl) was determined on MPA with the addition starch agar plates. The plates were incubated over night at 37°C. All experiments were performed in triplicate.

Molecular analysis

Molecular analysis in LAB was performed by molecular identification (16S rRNA gene sequencing) in GeXP Genetic analysis system (Beckman Coulter, USA). Over-night culture from strains of interest was used for total DNA isolation. DNA was isolated using commercial kit GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, CA, USA) following the

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kit procedure. For 16S RNA sequencing reaction following primers were used: LactoF: forward: 5'-TGGAAACAGRTGCATATACCG-3'; LactoR: reverse: 5'-GTCCATTGTGGAAGATTCCC-3'. For multiplication of 16S rRNA region following protocol was used: Reaction volume was 50 µl containing: 25 µl Red Taq Master Mix (VWR International), 2 µl forward primer, 2 µl reverse primer, 2 µl template DNA and 19 µl PCR grade water. PCR protocol was 35 cycles with following temperatures: 30 seconds at 95°C, 40 sec at 65°C; 30 seconds at 72°C and final elongation 5 minutes at 72°C. After PCR reaction PCR product was purified using PCR purification kit (Bionner, Korea) following the manufacturer's protocol. After purification gel electrophoresis was done. There was used 1.5% agarose gel (Merck, Germany). Cycle sequencing PCR reaction was done using DTCS Quick start kit (Beckman Coulter), following manufacturer's protocol. The protocol was: total reaction volume was 20 µl: 8 µl DTCS Quick Start Master Mix (Beckman Coulter), 2 µl Primer (forward/reverse); 5 µl PCR product and 5 µl PCR grade water. PCR protocol was: 30 cycles with following temperatures: 20 seconds at 96°C, 20 seconds at 50°C and 4 minutes at 60°C. PCR product was purified using ethanol precipitation. Preparation of fresh Stop Solution/Glycogen mixture of 2 µl of 3 M NaOAc (pH 5.2), 2 µl of 100 mM Na; 2 µl EDTA (pH 8.0) and 1 µl of 20 mg/ml of glycogen (supplied with the kit). To each of the wells, we add 5 µl of the Stop Solution/Glycogen mixture. Stop solution was prepared immediately before use and keep it at room temperature. The salt and glycogen facilitate precipitation of the DNA from the sequencing reactions. Precipitation is very inefficient without them and leads to low signal and potential color imbalance (low A signal may be observed) when run on the system. There were added 60 µl cold 95% ethanol/water (v/v) from -20°C freezer and mix thoroughly. Centrifuge at 14.000 rpm at 4°C for 15 minutes. The supernatant was carefully removed the with a micropipette (the pellet should be visible). The pellet was rinsed two times with 200 µl 70% ethanol/water (v/v) from -20°C freezer. For each rinse, centrifuge at 14.000 rpm at 4°C for a minimum of 2 minutes. The purpose of the washes is to remove the residual salts that remain after removing the supernatant from the precipitation step. Since salts are preferentially loaded by electrokinetic injection on the system, fewer sequencing fragments will be loaded. As the salts are removed, the pellet may become invisible. After second rinse with 70% ethanol the pellet was vacuum dried for 20 minutes for removing all the ethanol. After that, the samples were re-suspended with SLS buffer

(Beckman Coulter). After re-suspension samples were loaded into GeXP Genetic analysis system (Beckman Coulter, USA) using appropriate protocol.

Results and Discussion

Following the classical microbial methods were isolated 10 strains from 4 different natural sourdoughs prepared from wheat and rye flour.

The phenotypic analysis included study of cell morphology, spore forming, oxidative test and gas production on glucose. All isolated strains were Gram positive rods, non-spore forming, non-motile bacteria, and negative for catalase and oxidase tests.

The increasing interest in starter cultures for sourdough fermentations requires better insights into the genetic and phenotypic diversity of strains for exploitation in technological processes. Although still important for the description of new species, conventional methods such as carbohydrate fermentation pattern analysis and cell wall analyses are not reliable for the accurate identification of LAB at the species level. The use of 16S rRNA gene sequencing is generally regarded as a more reliable solution for the classification and identification of LAB, although the differentiation of closely related *Lactobacillus* species is not always straightforward, due to a high degree of conservation within this genus (Ehrmann & Vogel, 2005).

The used strains isolated from sourdough prepared from wheat and rye flour was sequenced for 16S rRNA. Results are shown in a Table 1. The data in the table show that in terms of differentiation 90% of the isolates are of the type *L. plantarum* and 10% are the type *L. helveticus*. In the terms of flour type, in the white wheat flours only *L. plantarum* species was isolated, whereas in the rye ones a *L. helveticus* species was isolated as well.

Proteolysis by lactic acid bacteria during sourdough fermentation has been poorly investigated. It may have repercussions on rheology and staleness free amino acids and small peptides are important for rapid microbial growth and acidification during fermentation and as precursors for flavor development of leavened baked goods (Di Cagno et al. 2002). Some fundamental questions still remain unanswered: they concern the capacity of lactic acid bacteria to hydrolyze water-insoluble proteins, such as gliadins and glutenins; the influence of dough acidification in modifying the wheat protein pattern and network; and the capacity of lactic acid bacteria to interfere with the generation of biologically active peptides which adversely affect the human intestinal mucosa,

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resulting in cereal intolerance. The isolated species were put to physiological analysis regarding the proteolytic and the amylolytic activity. The results from the analysis for proteolytic activity are shown on Figure 1.

From this data on Figure 1 that the species *L. plantarum* 4p, *L. plantarum* 2, and *L. plantarum* 2p demonstrated high proteolytic activity.

Today, the diverse group of LAB includes species that are among the best-studied microorganisms and proteolysis is one of the particular physiological traits of LAB of which detailed knowledge was obtained.

From the 10 isolates were screened for amylolytic activity but only 2 strains *L. plantarum* 5, *L. plantarum* 5p, and *L. plantarum* 6p presented high amylolytic activity and were qualified as amylase overproducing isolates. The amylolytic power was defined as the average diameter (mm) of starch hydrolysis halo (Figure 2) provoked by a stain after its inoculation in micro-well on MRS-starch agar plates for 48 h incubation at optimum temperature of growth for three assays.

Table 1. 16S rRNA determination of *Lactobacillus* strains.

Strain	Species	Identification (%)	Max score
1 forward primer	<i>Lactobacillus plantarum</i>	98%	1170
1 reverse primer	<i>Lactobacillus plantarum</i>	97%	1099
4 forward primer	<i>Lactobacillus plantarum</i>	97%	986
4 reverse primer	<i>Lactobacillus plantarum</i>	98%	1003
4p forward primer	<i>Lactobacillus plantarum</i>	97%	1234
4p reverse primer	<i>Lactobacillus plantarum</i>	96%	1136
5 forward primer	<i>Lactobacillus plantarum</i>	95%	1100
5 reverse primer	<i>Lactobacillus plantarum</i>	95%	1100
5p forward primer	<i>Lactobacillus plantarum</i>	97%	876
5p reverse primer	<i>Lactobacillus plantarum</i>	97%	854
6p forward primer	<i>Lactobacillus helveticus</i>	96%	1257
6p reverse primer	<i>Lactobacillus helveticus</i>	96%	1214



Figure 1. Plates assays for detection of proteolytic activity of LAB on skin milk agar plate medium. The diameter of halo was revealed after 48h of culture at 37°C.



Figure 2. Plates assays for detection of amylase activity of LAB on MRS-starch agar plate medium. The diameter of hydrolysis halo was revealed by flooding the plates with iodine solution (0.1% I₂ + 1% KI) after 48 h of culture at 40°C. 8 – *L. plantarum* 5; 9 – *L. plantarum* 6p; 10 – *L. plantarum* 5p; 11 – *L. plantarum* 4p; 12 *L. plantarum* 4; 13 – *L. plantarum* 2; 14 – *L. plantarum* 2p.

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The amylolytic power is an expression of the capacity of an isolate to degrade starch during the culture cultivation.

Sanni *et al.* (2002) described amylolytic strains *L. plantarum* and *L. fermentum* strains in various traditional amylaceous fermented foods (ALAB). ALAB are generally screened in fermented amylaceous foods.

Owing to their relatively high starch content, starchy biomass appears as an important eco-niche for the screening and isolation of ALAB, which can be industrially applied to convert starch into mono- and disaccharides for lactic acid fermentation. Most ALAB isolated belong to the *Lactobacillus* genus.

Because at industrial scale, the use of glucose addition is an expensive alternative, there is interest in the use of a cheaper source of carbon, such as starch, the most abundantly available raw material on earth next to cellulose. This, in combination with amylolytic LAB may help to decrease the cost of the overall fermentation process. Amylolytic LAB can convert the starch directly into lactic acid. Development of production strains which ferment starch to lactic acid in a single step is necessary to make the process economical (Fossi & Tavea, 2013).

This study has been able to further complement studies on the occurrence of amylolytic and proteolytic lactic acid bacteria from Bulgarian wheat and rye flour. The obtained results indicate that the proteolytic and amylolytic activities are specific in terms of species and strains.

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