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TRANSIT TOLERANCE OF BULGARIAN DAIRY LACTOBACILLI TO ACIDIC PH AND BILE SALTS

R. Tropcheva, S. Danova*

Department of Microbial Genetics, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

ABSTRACT

Widely acceptance of probiotic concept strongly stimulates the scientific research on lactic acid bacteria. Several *in vitro* criteria were developed, in order to select appropriate *Lactobacillus* strains with probiotic properties. The viability of lactobacilli under simulated conditions of the gastrointestinal tract - high acidity and bile salts is widely accepted criterion for the probiotics selection. Four strains from the laboratory collection - *Lactobacillus brevis* SR3, *Lactobacillus brevis* SR53, *Lactobacillus plantarum* SR3C and *Lactobacillus plantarum* SR11, pre-selected as active antagonists of food pathogens, were evaluated for their transit tolerance *in vitro*. Initially, they were identified at a species level using conventional microbiological approaches and PCR techniques. The acid and bile salts tolerance of the tested lactobacilli were estimated spectrophotometrically and through control cultivations on agar plates. All tested strains survived in acidic conditions, even after 48-h cultivation. The exposure to different concentration of oxgall (0.5, 1 and 2 % w/v) was less detrimental than the exposure to pH 2.0. The strains *L. brevis* SR3, *L. brevis* SR53 and *L. plantarum* SR11 were selected as bile- and acid-resistant, which are able to survive the harsh conditions in gastrointestinal tract.

Key words: Bulgarian dairy lactobacilli, tolerance, low pH, bile salts

INTRODUCTION

Bulgaria is a country with a long tradition in the production of different fermented milk products. Their natural lactic acid microflora contributes significantly to the development of specific sensory properties and it is responsible for prolonged shelf-life and food preservation (1). However, a limited data exists on benefits and functions of non-starter lactic acid bacteria (NSLAB) from Bulgarian dairy products. Some probiotic and technologically relevant properties of lactobacilli, isolated from Bulgarian artisanal cheeses have been estimated (2). Moreover, several foods originated lactic acid bacteria (LAB) have been characterized as effective antagonists against different food pathogens and fungi (3, 4). They were pre-selected for further characterization as potential probiotics. The ability to survive in the gastrointestinal tract (GIT) is one of the main desirable characteristics required for an effective probiotic action (5, 6). In addition, according to the guidelines for the evaluation of probiotics in food reported by a Joint FAO/WHO working group (7), two of the currently most widely used *in vitro* tests are resistance to gastric acidity and bile salts, as based on both survival and growth studies. LAB should retain its vitality until it reaches the place intended for its effectiveness. GIT is a complex and dynamic system that includes a range of different physiological and physicochemical processes that take place in the stomach, small intestine and colon (8). These conditions are very harmful to living microorganisms. Low pH in stomach is the first barrier that a probiotic strain has to overcome when it is in the GIT. The bile

***Correspondence to**: *Svetla Danova, e-mail: std@microbio.bas.bg, Department of Microbial Genetics, The Stephan Angeloff Institute of Microbiology, Bulgarian Acad. Sci.,26, Acad. G. Bontchev, str, 1113 Sofia, Bulgaria, Fax: + 359 2 8700109*

salts are the next obstacle. As of today, the mechanisms of survival to bile exposure of lactobacilli are not fully understood (9) and more investigations have to be done.

With this aim, the tolerance of four Bulgarian *Lactobacillus* strains to low pH and bile salts was estimated *in vitro*.

MATERIALS AND METHODS

Lactobacillus strains:

Four LAB strains from our laboratory collection were included in the present study (SR3, SR53, SR11, SR3C). They were isolated from home-made milk products (without an addition of any starter cultures), collected from the Rila and Rodopa mountain regions of Bulgaria. The pure LAB cultures were isolated on selective agar media: Rogosa (*Difco*, USA), APT (*Difco Detroit* Michigan, USA) and L-S differentiation arap (*Scharlau*, Spain) after cultivations at 30, 37°C and 42°C in anaerobic conditions (BBL®*Gas Pak Anaerobic System Envelopes*). Initial agar-diffusion tests allowed their pre-selection as antagonists with a broad spectrum of activity.

Identification of LAB isolates:

The identification of the newly isolated LAB was based on conventional microbiological approaches and PCR techniques. The protocol of Guarneri (10) was applied in PCR analysis with species-specific primers for *Lactobacillus brevis.* The multiplex PCR for *Lactobacillus plantarum* group was done according to Torriani et al. (11).

Acid pH and bile salt tolerance:

Exponentially growing LAB cultures were inoculated in MRS broth with pH 2.0 and pH 4.0 (adjusted with 37 % HCl before sterilization) and in a modified MRS broth pH 6.0 with different concentrations of Oxgall (0.5, 1 and 2 % w/v; Merck, USA). MRS broth pH 6.0 without supplements was used as a control medium. All samples were incubated anaerobically at 37°C. The tolerance of the tested strains was estimated spectrophotometrically by the change in their optical density ($OD_{595\ nm}$) during the cultivation. Each assay was performed in triplicate.

RESULTS

An original protocol, combining selective media and different cultivation conditions allowed isolation of several LAB culture from collected artisanal dairy products. By preliminary tests against foodborne pathogens were selected four strains - SR3, SR53, SR11 and SR3C with a broad spectrum activity. The strains were characterized as Gram (+), catalase (-), non-sporeforming rods and were identified to the species level by classical biochemical tests and PCR techniques. The positive results from species-specific PCR analyzes (according to 10 and 11), (data not shown) confirmed the initial affiliation of SR3 and SR53 to species *L. brevis* and SR11 and SR3C to species *L. plantarum*.

The assessment of resistance to acidity is necessary, as the secretion of gastric acid constitutes a primary defense mechanism against most ingested microorganisms. Before reaching the intestinal tract, probiotic bacteria must survive transit through the stomach. *In vitro* tests with the selected four lactobacilli showed a strain-specific viability at pH 2.0 and pH 4.0 **(Figure 1)**. An increase in OD after 48 hours cultivation in MRS (pH 4.0) was observed for all tested lactobacilli **(Figure 1B)** and for the strain SR3 was even measured a slight increase in OD after 48 hours at pH 2.0 **(Figure 1A)**. The parallel application of a standard agar cultivation method confirmed the good viability and survival at pH 2.0 for the other strains too **(Figure 1A)**.

Resistance to bile salts is considered an important property in strains envisaged as probiotics. The growth inhibition following exposure to different concentrations of bile salts was detected for the four strains, selected as resistant to pH 2.0 and pH 4.0 **(Figure 1).** The exposure of our LAB strains to different concentration of oxgall - 0.5, 1 and 2 % **(Figure 2)** was less destructive than the exposure to pH 2.0. Even more, for strain *L. brevis* SR53 **(Figure 2B)** and *L. plantarum* SR11 **(Figure 2C)** were detected an increases in OD after 48 hours cultivation in MRS broth pH 6.0 with 2% oxgall, compared to their OD in the control samples – MRS broth pH 6.0 without oxgall.

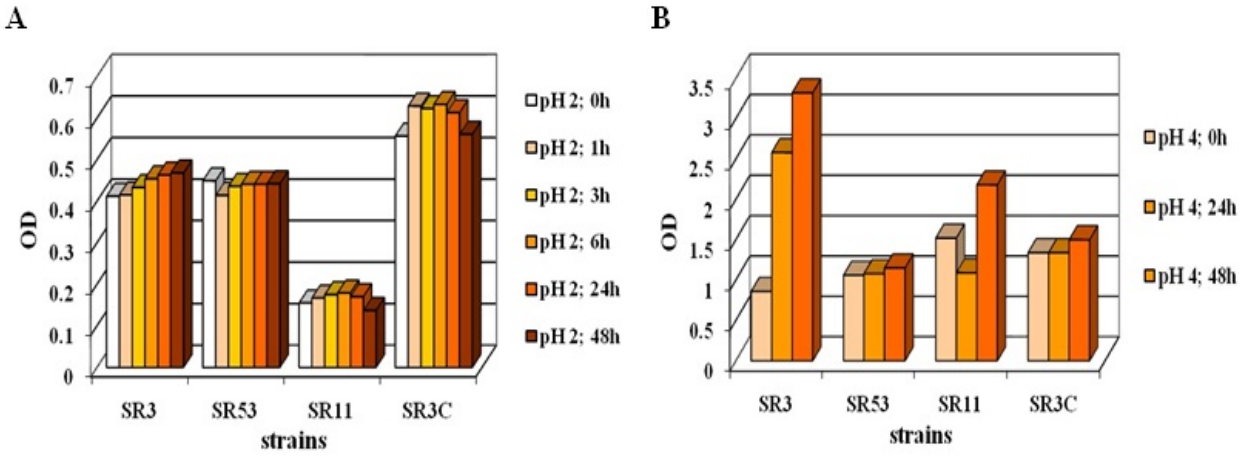

Figure 1. Effect of pH 2.0 (A) and pH 4.0 (B) on the viability of four lactobacilli isolated from fermented milk products.

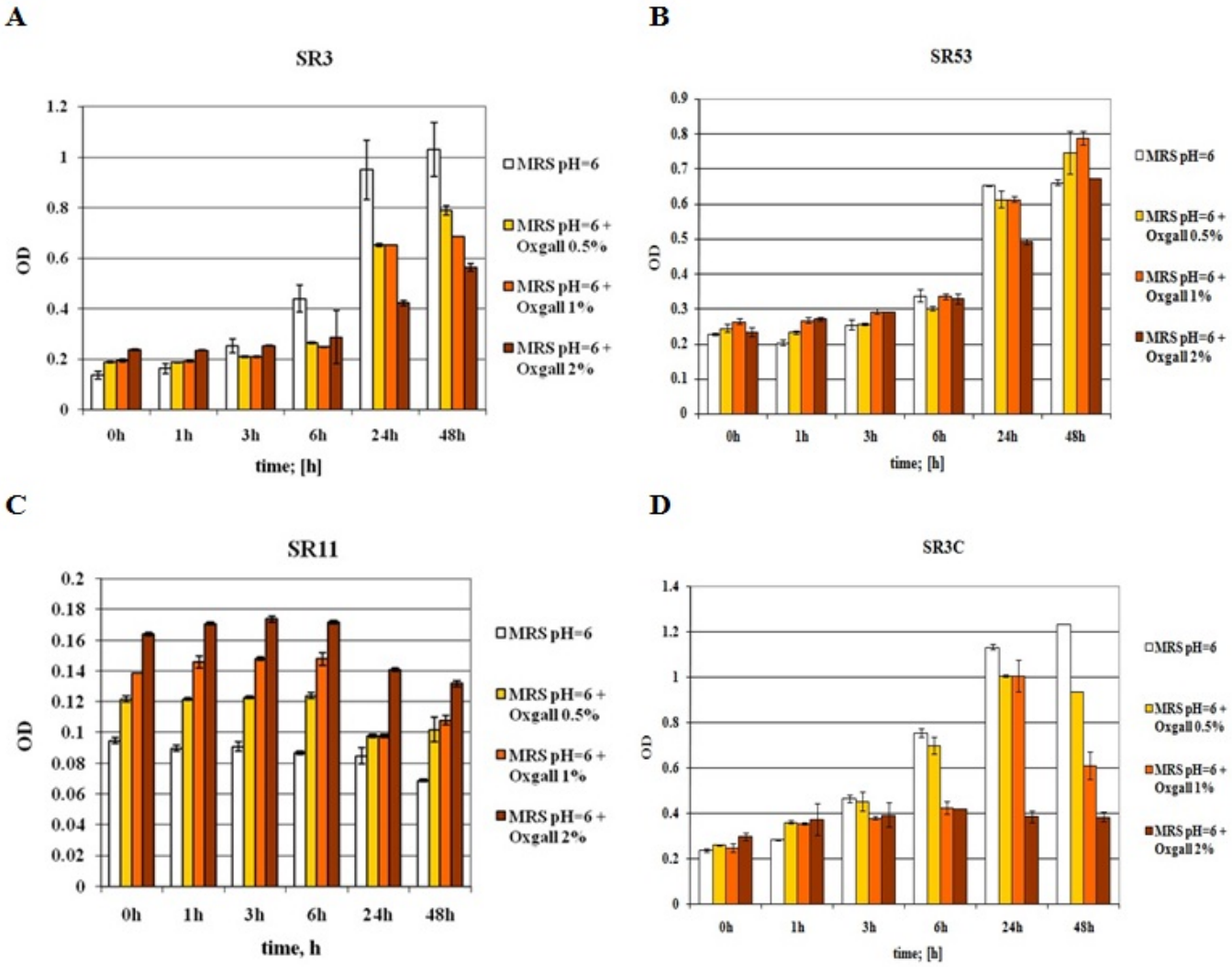

Figure 2. Effect of different concentration of oxgall (0.5; 1 and 2 % w/v) on the growth of pre-selected as acid tolerant lactobacilli: A) *L. brevis* SR3, B) *L. brevis* SR53, C) *L. plantarum* SR11, D) *L. plantarum* SR3C.

DISCUSSION

To express their positive effect on human health, the active LAB strains should be able to survive the harsh conditions in GIT. There are reports on maintained viability in MRS broth with pH 4.0 for 3-h test period, but at the same time a decreased number of viable bacteria after 3-h incubation in MRS broth with pH 2.0 (12). According to Charteris et al. (13), enteric lactobacilli are able to tolerate pH 2.0 for several minutes. Our dairy strains survived in acidic conditions, even over 48 hours (Figure 1), estimated after a control cultivations of aliquots (100 µl of each culture of appropriate dilutions) on MRS agar (data not shown). A good viability and resistance at pH 2.0 was established for all of the testad strains (Figure 1A). Similar results were reported by Dunne et al. (14) and El-Naggar (15).

The *in vitro* assessment of bile acid resistance is necessary when evaluating the potential of using lactic acid bacteria as effective probiotics (16). However, there is still no consensus about the precise concentration to which the selected strain should be tolerant. The physiological concentration of bile acids in the small intestine is between 5000 and 20 000 µM (17). In this study, concentrations of 0.5% bile salts (equivalent to 12 255 µM), 1% (24 510 µM) and 2% (49 020 µM) were used, which are higher than the concentrations of 0.3% or 0.15% that have previously been used by other investigators of bacterial probiotic properties (18 - 20). The 2% oxgall (bile salt) used for testing our strains represents the extreme concentration obtained in animal or human intestine during the first hour of digestion (21). Elina Rönkä et al. have reported that the low concentration of bile salts in the growth medium had no effect on the LAB viability (12), but according to other authors there was a decline in viable counts of some lactobacilli after culturing in MRS broth with bile salts (22). These reports sujests that the resistance to bile salts is a strain-specific feature. The tested LAB strains demonstrated a better resistance and tolerance to the different concentrations of bile salts compared to the tests with pH 2.0 (Figure 1A and 2). The most promising results even after a 48-h cultivation in MRS broth pH 6.0 with 2% oxgall showed *L. brevis* SR53 **(Figure 2B)** and *L. plantarum* SR11 **(Figure 2C)**. The observed differences between the tolerance of the species *L. plantarum* and *L. brevis* and between their strains (*L. brevis* SR3, SR53 and *L. plantarum* SR11, SR3C) (Figure 2) are in accordance with the statement that the resistance to bile salts varies a lot among the LAB species and even between strains themselves (23). All this confirms the strain specific nature of the bile stress response (24).

The strains *L. brevis* SR3, SR53 and *L. plantarum* SR11 were selected as bile- and acid-resistant isolates (Figures 1 and 2), which are able to survive in the environment of gastrointestinal tract. They will be studied additionally in a dynamic model of GIT, because as it has been pointed out by various researchers in the field (14,25-27), *in vitro* studies can only partiallymimic the actual *in situ* conditions in GIT and GUT. *In vivo*, the transit time through the stomach can be less than 1 hour or up to 3-4 hours depending on individual and the specific conditions. Therefore, retention of viability for at least 3 hours may be considered as a sufficient criterion for a probiotic pre-selection. Our strains demonstrate a good stability and viability even after 48 h passage. Similar results were reported by other authors. Hammes et al. have showed that the LAB involved in the production of various fermented foods have the potential to survive in the GIT and also the microorganisms are capable to synthesize useful substances (28).

CONCLUSION

The tests, in the present work are based on the hostile gut environment, which they mimic under *in vitro* conditions. In most cases in the practice, the application of probiotic strains is often made in a form of supplements, or LAB are immobilized on a matrix. This protects the bacteria and they are not directly exposed to these detrimental effects of acidity and bile, as in our experiments. Therefore, from the tested strains could be expect a better survival in the real conditions of GIT and they can be classified as potential beneficial cultures. The evaluated as candidate probiotics lactobacilli, based on these tests, should be subjected to an additional validation in appropriate *in vivo* models.

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