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PRELIMINARY SCREENING OF PROBIOTICS CHARACTERISTICS OF BACTERIA ISOLATED FROM DIFFERENT KIND OF DOMESTIC CHEESE

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Abstract. *Probiotics are beneficial bacteria that contribute to the health and balance of the intestinal tract. In people, the most common way to prevent from getting any infectious disease or digestive upset is by using vaccines and antibiotics. Besides that, naturally occurring microflora is a good alternative to protect the host gut. In developing a good strain of probiotics, there are a few criteria to be achieved by the bacteria such as ability to tolerate acidic condition and survival in the presence of bile salts. All isolates were grown 24 hours in Man Rogosa and Sharp (MRS). Acid tolerance test was done by incubating the isolates into Phosphate Buffered Saline (PBS) in pH 2, and 3 for 3 hours. For the bile salts test, the isolates were exposed to the bile salts for 4 hours. At each hour, the optical density (OD) of the isolates was measured. For both test, the cells survival in these two conditions was measured by OD after incubation on new fresh MRS. From 12 isolates, 6 isolates are acid tolerate while 5 isolates are able to survive in presence of bile salts. For this preliminary screening, 4 isolates were selected for further works on probiotics characteristics.*

Keywords*: probiotics; protection; beneficial; screening.*

1.INTRODUCTION

Probiotics are known as one of the most beneficial microbial feed supplement to the animal. It is significant to the animal host in improving their gastrointestinal health by accelerating the production of the beneficial microflora, enhancing the host resistance from pathogenic and toxic microorganisms as well as increasing the level of immunomodulatory of the host (Mourad and Nour-Eddine, 2006).

Many beneficial effects of these microorganisms including anti-inflammatory properties, modulation of host's immune responses, reduction of lactose intolerance as well as inhibition of pathogenic bacteria have been described (De Vrese and Schrezenmeir, 2008; Leahy et al., 2005). A suitable probiotics strain must tolerate acidic pH of stomach and bile salts of intestinal tract and be able to adhere to mucosal surfaces (Shobharani and Agrawal, 2011).

Traditionally, probiotics have been utilised in dairy products such as milk or yoghurt and it has been hypothesized that milk enhances probiotic efficacy by providing lactose as a substrate (Varcoe et al., 2002). At the present, a large number of dairy products are present on the market and are being promoted with health claims based on several characteristics of selected strains of lactic acid bacteria, particularly belonging to the genera Lactobacillus and Bifidobacterium (Shah, 2000).

Microorganisms ingested with food begin their journey to the lower intestinal tract via the mouth and are exposed during their transit through the gastrointestinal tract to successive stress factors that influence their survival (Marteau et al., 1993; Simon and Gorbach, 1987). The time reported from entrance to release from the stomach is about 90 min (Berrada et al., 1991), but further digestive processes have longer residence times. Cellular stress begins in the stomach, which has pH as low as 1.5 (Lankaputhra and Shah, 1995). Bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes, whose major components are lipids and fatty acids and these modifications may affect not only the cell permeability and viability, but also the interactions between the membrane and the environment (Gilliland, 1987; Gilliland et al., 1984). Therefore before a probiotics can benefit human health it must fulfil several criteria such as the ability to tolerate acid and bile salts as well as to grow in the lower intestinal tract (Zhu et al., 2000; Pereira and Gibson, 2002; Ouwehand et al., 2002; Hirayama and Rafter, 2000). So, the first tool in the selection of a strain of probiotics interest is represented by in vitro methods aiming to ascertain the ability to survive passage through the upper gastro-intestinal tract and arrive alive at its site of action.

In order to develop the probiotics as a feed supplement, a preliminary screening on the characteristics of the strain is important to determine its ability to survive in the gastrointestinal tract condition. The preliminary screening was done to select the best strain that could survive in acidic condition as well as in the presence of bile salts.

2. MATERIALS AND METHODS

2.1. Cheese collection

Three traditional cheese samples (~200 g) were collected from three traditional Macedonian families who produce cheese in domestic conditions. The samples were aseptically transferred to the Microbiological Lab, Department of Microbiology and Microbial Biotechnology, at theFaculty of Natural Sciences and Mathematics, "Ss. Cyril and Methodius" University, Skopje, North Macedonia, under cold and aseptic conditions.

2.2. Isolation of beneficial microbes

An amount of 25 g of each cheese sample was added to 225 ml 0.1% w/v peptone water and homogenized at 280 rpm for three min. The cheese suspension was diluted in 2% w/v sodium citrate and cultured on two De Man, Rogosa and Sharpe (MRS) agar plates and incubated under anaerobic conditions for 2 days at 37°C. The 3–4 different single colonies were randomly selected from each cultured plate. The selected colonies were Gram stained, examined microscopically, and catalase test was also performed. Gram-positive and catalase-negative bacilli were chosen and stored in cryotube containing 15% (v/v) glycerol at −20°C for further characterization.

2.3. Acid and bile tolerance

Tolerance to acid was determined according to the method described at Yu et al. (2013), as the following: isolated strains were grown in MRS broth at 37ºC for 24-48 h, and sub-cultured in fresh MRS broth adjusted to pH 2.0 and 3.0 with hydrochloric acid (3.0 mol/L). The initial bacterial concentration was adjusted to 10^9 CFU/mL and the survival rate was determined after incubation for 180 min at pH 2.0 and pH 3.0 which reflects the time spent by food in the stomach (Maragkoudakis et al., 2006). Survival rate was determined using plating on MRS agar and calculating viable counts at different intervals.

Bile tolerance test was conducted using the method described by Gilliland et al. (1984). Overnight cultures of the isolated strains with initial concentration of 10^9 CFU/mL were inoculated into MRS broth containing 0.5, 1.0 and 2.0 % (w/v) bile. Survival rate of the isolates were measured following 24 h incubation at 37°C using the method described for acid tolerance assay.

2.4. Antibiotic susceptibility

Susceptibility of the isolated strains to the antibiotics commonly used by human was evaluated according to the method described at Le Blanc et al. (2010). Briefly, the cultures were overlaid on Muller-Hinton agar plate and antibiotic discs were placed on it and incubated 24 h at 37°C. The assay was performed in triplicates and mean diameter of inhibition zones around antibiotic discs were recorded. The susceptibility was expressed in terms of resistance (R), intermediate susceptibility (I), and susceptibility (S) based on data from the National Committee for Clinical Laboratory Standards (NCCLS).

2.5. Haemolytic activity

In order to evaluate hemolytic activity of the isolates, fresh bacterial cultures were plated on Blood Agar plate containing 5% v/v horse blood. The plates were examined for signs of β- hemolysis (clear zones around colonies), α-hemolysis (green-hued zones around colonies) or γ- hemolysis (no zones around colonies) following 24-48 h incubation at 37°C (Zoumpopoulou et al., 2008).

2.6. Antimicrobial activity against Gram negative pathogen

Antimicrobial activity of the isolated strains against *S. enterica* serovar Typhimurium ATCC 14028 was investigated using the method described by Yu et al. (2013) and Ebrahimi et al. (2013). A fresh culture of the isolated bacterium, grown in MRS broth, was centrifuged (6000 g, 10 min, 4°C) and the resulting supernatant was adjusted to pH 6.5 with NaOH (1 M) in order to rule out acid inhibition. The supernatant was used to determine antibacterial activity of the isolated strain against *S. typhiurium* using well diffusion assay. This assay was performed in triplicates and mean diameter of inhibition halos were measured.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of potent probiotic strain

Twelve bacterial strains were isolated from three traditional cheese samples from N. Macedonia. Further studies were based on the morphology, Gram staining, Catalase production and motility tests. They were Gram positive, Catalase negative, non motile rods and were regarded for further characterized as potent probiotic strains.

The growth was observed at 10, 37 and 45ºC but the strains did not tolerate 55 ºC. In addition, the culture grew well in the media containing 2.5, 6.5 and 10% salt but generally did not grow in the medium containing 18% NaCl. Tables 1 and 2 displays results from these tests.

Table 1. Morphological and physiological characterization of the isolates.

Isolate No.	growth on MRS agar	morphology	Gram staining	catalase test	motility test
Isolate No.1	+	rods	+	-	-
Isolate No.2	+	rods	+	-	-
Isolate No.3	+	rods	+	-	-
Isolate No.4	+	rods	+	-	-
Isolate No.5	+	rods	+	-	-
Isolate No.6	+	rods	+	-	-
Isolate No.7	+	rods	+	-	-
Isolate No.8	+	rods	+	-	-
Isolate No.9	+	rods	+	-	-
Isolate No.10	+	rods	+	-	-
Isolate No.11	+	rods	+	-	-
Isolate No.12	+	rods	+	-	-

According to Peterson et al. (1990) and Salminen et al. (1996) only few microorganisms survive in cheeses because of its low redox, low pH and high salt. In our research only three isolates were grown on 18% salt in the medium, but all tested isolates grown on 2.5, 6.5 and 10% of salt.

Table 2. The tolerance of isolated strains of different incubation temperature and concentration of salt in medium.

Isolate No.	incubation temperature (ºC)				concentration of salt (%)			
	10	37	45	55	2.5	6.5	10	18
Isolate No.1	+	+	+	-	+	+	+	-
Isolate No.2	+	+	+	-	+	+	+	-
Isolate No.3	+	+	+	-	+	+	+	-

Isolate No.4	+	+	+	+	+	+	+	-
Isolate No.5	+	+	+	-	+	+	+	-
Isolate No.6	+	+	+	-	+	+	+	-
Isolate No.7	+	+	+	-	+	+	+	-
Isolate No.8	+	+	+	-	+	+	+	+
Isolate No.9	+	+	+	+	+	+	+	-
Isolate No.10	+	+	+	-	+	+	+	-
Isolate No.11	+	+	+	-	+	+	+	+
Isolate No.12	+	+	+	+	+	+	+	+

3.2. Acid and bile tolerance

From twelve isolates, six isolates are acid tolerate (for pH 2, and pH 3) while others did not grow on acidic medium. Acid tolerance assay showed that only isolates No. 1, 3, 5, 7 and 9 can tolerate both pH, whith survival percentage between 58 and 80% (for pH 2) and survival rate beween 65 and 85% (for pH 3).
Bacterial isolates were treated with different concentrations of bile salts. According to the results, from tested isolates five strains are able to survive in presence of bile salts. The results showed decrease in viability by increasing bile concentration.

Table 3. Results from acid tolerance test, bile tolerance test, antimicrobial activity test and haemolysis test.

Isolate No.	Acid tolerance test		Bile tolerance test			Antimicrobial activity test ZI (mm)	Haemolyis test
	pH 2	pH 3	0.5%	1%	2%		
Isolate No.1	+	+	+	+	+	+ (5 mm)	γ (no haemolysis)
Isolate No.2	-	-	-	-	-	-	α
Isolate No.3	+	+	+	+	+	+ (7 mm)	γ (no haemolysis)
Isolate No.4	+	-	-	-	-	-	α
Isolate No.5	+	+	+	+	+	+ (10 mm)	γ (no haemolysis)
Isolate No.6	-	-	-	-	-	-	β
Isolate No.7	+	+	+	+	+	+ (11 mm)	γ (no haemolysis)
Isolate No.8	-	-	+	+	+	+ (2 mm)	β
Isolate No.9	+	+	-	-	-	-	β
Isolate No.10	-	+	-	-	-	-	β
Isolate No.11	-	-	-	-	-	-	α
Isolate No.12	-	-	-	-	-	-	α

For a probiotics strain, survival under gastrointestinal environment condition is important criteria to be fulfilled which depends on tolerance to low pH and high bile concentration as well as resistance to antibiotics and antimicrobial activity against gram negative pathogens, such as *S. typhimurium*. Also, tolerance to extremely acidic condition is an important feature of a probiotics strain (Guo et al., 2009).
Our tested isolates from domestic Macedonian cheese showed good tolerance to low pH showing its ability to survive under acidic environment of stomach. These strains were more tolerant to low pH in comparison with the *L. plantarum* studied by Lotfi et al. (2010) who isolated LAB from traditional cheese from Heris and Sarab regions.
The physiological concentration of bile in the small intestine has been reported to be between 0.2 and 2.0% (Gunn, 2000*)*.Our isolates from this study showed a good tolerance to higher bile salts concentrations. Also, the good bile tolerance of the isolated LAB strains has been reported previously at García-Ruiz et al. (2014). Similar results were

also reported by Mourad and Nour-Eddine (2006) who found that one of their isolates showed 65% survival rate following exposure to 2.0% bile. Resistance to bile salts varies a lot among lactic acid bacteria species and even between strains themselves. Bile resistance of some strains is related to the specific enzymatic activity of Bile Salt Hydrolase (BSH) which helps hydrolyzing conjugated bile, thus reducing its toxic effect (Du Toit et al., 1998).

3.3. Antimicrobial activity against Gram negative pathogen

Antibacterial activity is an important feature of the probiotics strains. The isolates were checked for their antibacterial activity against main gastrointestinal pathogen *S. typhimurium*. Our results showed that five isolates inhibit indicator pathogen bacteria with different inhibition level. Isolate No.8 was weakly effective against *S. typhimurium* with inhibition zone diameter of 2 mm, isolate No.1 was slightly more effective against *S. typhimurium* with inhibition zone diameter of 5 mm, whereas other three isolates (No. 3, 5 and 7) showed stronger antibacterial activity against tested Gram negative pathogen, with inhibition zone diameter of 7 (isolate No. 3), 10 (isolate No. 5) and 11 mm (isolate No. 7).

Antimicrobial activity of LAB against potential pathogens has been reported by Yu et al. (2013), while some studies did not show effective antibacterial activity of tested LAB strains against pathogenic bacteria (Maragkoudakis et al., 2006). This property of the isolated bacterium can be used in prophylactic or therapeutic usage. The inhibitory activity of the LAB strains might be due to either production of organic acids or bacteriocines (Yu et al., 2013).

3.4. Haemolytic activity

Absence of hemolytic activity is another considered safety prerequisite for the selection of a probiotics strain. Hemolysis is the break down of the membrane of red blood cells by a bacterial protein known as hemolysin, which causes the release of hemoglobin from the red blood cell.

The hemolytic activity of isolates was determined by using Blood agar containing 5% (w/v) horse blood and the plates were incubated at 37 °C for 48 h. After incubation, the hemolytic activity of isolated strains was evaluated and classified on the basis of lysis of red blood cells in the medium around the colonies. The green zones around colonies (α-hemolysis), clear zones around colonies (β-hemolysis) and no zones around colonies (γ-hemolysis) on Blood agar plates. Only strains with γ-hemolysis are considered as safe (Mangia e al., 2019).

Four of the tested strains showed α-hemolytic acivity, the other four isolates were with β-hemolytic activity when grown on Blood agar plates. Only isolates No. 1, 3, 5 and 7 showed γ hemolytic, i.e., negative, or no hemolytic activity. These results confirm their safety as potent probiotics strains.

3.5. Antibiotic susceptibility test

Frequent antibiotic administration causes gut microbiota imbalance and an increased susceptibility to infection was caused by opportunistic microorganisms (Willing et al., 2011). Probiotics strains which are resistant to antibiotics can proliferate in gut and maintain microbial balance and reduce opportunistic microorganisms (Le Blanc et al., 2010).

The antibiotic susceptibility of isolates was tested against selected antibiotics (erythromycin, streptomycin, kanamycin, vancomycin, penicillin, gentamicin and polymyxin B) commonly used by human, by using antibiotic disc diffusion method on Muller-Hinton agar plates. Antibiotic discs were placed on inoculated and solidified Muller-Hinton agar platea and give them 30 min for antibiotic diffusion and after that incubated (37 °C for 48 h). The zone of inhibition was measured for each antibiotic disc after the completion of incubation. The susceptibility was expressed in terms of resistance (R), intermediate susceptibility (I), and susceptibility (S) based on data from the National Committee for Clinical Laboratory Standards (NCCLS). The test was performed only with isolates No. 1, 3, 5 and 7, which were with γ hemolytic activity on previous test.

Table 4. Susceptibility of the isolated strains to different antibiotics.

antibiotics	concentration	zone of inhibition (mm)				susceptibility			
		No1	No3	No5	No7	No1	No3	No5	No7
erythromycin	15 μg	20	20	11	9	I	I	R	R
streptomycin	10 μg	15	21	5	6	S	S	R	R
kanamycin	30 μg	11	16	18	9	R	I	I	R
vancomycin	30 μg	13	10	15	16	R	R	R	R
penicillin	10 IU	29	34	20	18	S	S	I	I
gentamicin	10 μg	14	19	19	13	I	S	S	I
polymyxin B	300 IU	8	7	5	7	R	R	R	R
R= **R**esistance, **I**= **I**ntermediate susceptibility, **S**= **S**usceptibility									

From all four tested isolates, isolate No7 showed resistance to majority of antibiotics used in this study, including erythromycin, streptomycin, kanamycin, vancomycinand polymyxin B. In contrast, isolate No3 from our study was susceptible to penicillin, gentamicin as well as streptomycin. Table 4 compares antibiotic resistance of all four isolates.

Similar antibiotic resistance pattern of probiotic *L. plantarum* strains was reported by Yu et al. (2013). The ability of probiotics to resist antibiotics might be beneficial to people suffering from intestinal disorders due to improper administration of antibiotics (Salminen et al., 1998). However, it is important that the bacterial strains involved do not transfer antibiotic resistance genes from foods to intestinal microflora (Mathur and Singh, 2005).

4. CONCLUSIONS

In this study, macedonian domestic cheeses were examined to isolate potentially probiotics bacteria for the first time and a four strains with good probiotics properties were isolated. These isolates showed tolerance to high bile concentration, low pH and survived under condition simulating human gastrointestinal tract. Thus, it could be predicted that isolates would be able to pass stomach and reach intestine in adequate amounts. In addition, this strain displayed a good antibacterial activity against Gram negative food-borne pathogens *S. typhimurium*. Thus, these four isolates could be considered as a good probiotics candidate. However, further investigations including *in vivo* experiments as well as molecular analysis would be helpful to elucidate its potential health benefits and application as a probiotics strains in the dairy industry.

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