Research article

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An investigation on the probiotic properties of *Lactobacillus fermentum*

Serap Çetinkaya[1]*, Samet Kocabay[2], Ali Fazıl Yenidünya[1]

ABSTRACT

The study assessed some of the probiotic characteristics of an isolate (AB4 29) of *Lactobacillus fermentum*. AB4 29 was isolated from a faecal sample of an infant, fed with breast milk. The results indicated that the isolate had acceptable survival rates in gastric juice both in the presence and in absence of pepsin. It also displayed acceptable sensitivity rates to eleven different antibiotics. Hydrophobicity test showed that the isolate had a good capacity to adhere to xylene. It could also destroy sodium salts. AB4 29 displayed the least survival rates in bile salt. These initial findings could suggest that infant faeces and breast milk could serve as good sources of probiotic organisms.

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Introduction

Currently, many of the commercial probiotic strains are obtained from the intestines of healthy infants and adults. Current research therefore has mainly focused on the identification of those bacteria which could survive in the gut and compete with other microorganisms [1]. In the selection of probiotic strains, the main consideration is their beneficial properties to humans. The niche of these bacteria is the column. Probiotics are usually administered by the mouth and thus they must survive the harsh conditions while passing through the gastrointestinal tract (GI) to arrive at this final destination. Therefore, they are expected to resist salivary enzymes, stomach acid, and bile acids and salts, on the way. The cells of a probiotic strain should also form aggregates with themselves as well as with other microorganisms. The self-clusters form a biological barrier in the cavities of the GI- and

[1] Department of Molecular Biology and Genetics, Science Faculty, Sivas Cumhuriyet University, Sivas Turkey.
[2] Inonu University, Science and Literature Faculty, Department of Molecular Biology and Genetics, Malatya, Turkey
Corresponding Author**:** Serap Çetinkaya, e-mail: serapcetinkaya2012@gmail.com

urogenital systems, whereas the heterogeneous accumulations are necessary for the neutralisation of other bacteria [2-4].

Many probiotic microorganisms have been derived from genera known as lactic acid bacteria (LAB). They are obtained from food sources and therefore are generally known as safe (GRAS). Species of *Bifidobacterium* and *Lactobacillus* include many of the current probiotic strains. They are Gram (+), and their morphology can be rod, spherical or both. They do not form spores. They are relatively more resistant to stomach acids, and to lysozyme [5, 6]. Yeasts such as members of *Saccharomyces*, have also been used as probiotics [7].

Main metabolites produced by LABs include bio surfactants, carbon dioxide, di-acetyl, H_2O_2, lactic acid, and protein compounds (bacteriocin and bacteriocin-like substances) and they generally have antimicrobial activities [8-10].

Lactic acid is a sour and odourless fermentation product [11]. During growth LABs also produce hydrogen peroxide. It is a thermodynamically unstable compound and readily decomposes into water and oxygen. The amount of H_2O_2 produced differs from genus to genus, between species, and even among the strains of the same species [12, 13].

The aim of the study was to demonstrate whether infant faeces would be a source for the probiotic bacteria. Thus faecal samples collected belonged to the infants who were born by natural means and fed with breast-milk only. Thus the first step involved the isolation and identification of the LAB members and the second step included the studies performed for the investigation of some of their probiotic characteristics. Probiotic properties were screened by growing the isolated strain in simulated gastric- and intestinal juice, and in the presence of antibiotics. In addition, surface hydrophobicity was also determined.

Materials and Methods

Isolation and identification of the faecal isolate

One-gram faecal sample was inoculated in 10mL liquid culture and grown for 12h at 37°C (MRS, pH 6.3: 2% glucose, 0.2% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1% meat extract, 0.005% $MnSO_4 \cdot 4H_2O$, 1% peptone, 0.5% sodium acetate, 0.5% yeast extract, 0.2% tri-ammonium citrate). Colonies were obtained and purified by the streak method and by successive passages on MRS-agar media. Bacterial stocks were prepared in 20% glycerol. The isolates

were screened for Gram reaction and catalase activity. Gram (+) and catalase negative isolates were saved at -80^{o}C for further use.

For the molecular characterisation, DNA was prepared and used for the amplification of 16S rRNA gene by using the method described in Bulut *et al*. (2004) [14]. Amplification was carried out in a Thermo Cycler System (Thermo Electron Corp., USA). Nucleotide sequence of the two oligo-primers used for the amplification were forward, 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse, 5′-CTACGGCTACCTTGTTACGA-3′ [15]. Final reaction volume (50µl) included 200ng DNA template, 0.2mM dNTPs, 1.5mM $MgCl_2$, 10pmol each of the oligomers in 1x PCR buffer (10mM Tris–HCl, 50mM KCl, 8g Nonident P40/l, pH 8; MBI Fermentas, Lithuania), and 1.25 U Taq DNA polymerase (MBI Fermentas). Amplification reactions included 5 min initial denaturing step at 94^{o}C; each of the 40 amplification cycles included 1 min denaturation step at 94^{o}C, 1 min annealing at 58^{o}C, and 1 min elongation at 72 ^{o}C Amplification ended with a 10 min terminal extension step at 72 ^{o}C.

Determination of probiotic properties of the isolates

Tolerance to low pH

Four MRS broth media with different pH (2, 3, 4, and 6.3) were prepared. Strain AB4 29, pH 6.3 (control) was inoculated into MRS broth and grown overnight in a shaking water bath at 37^{o}C. The culture was divided into four sterile falcon tubes and the cells were precipitated for 10 min at 5,000 rpm at 4^{o}C. After removing the supernatant, cells were resuspended in one of the MRS broths (10ml) with different pH [pH 2, pH 3, pH4, and pH 6.3 (control)].

Sequential dilution (10^{12}-fold) was performed in sterile 4.5 ml NaCl (0.85%). One hundred microliters of the last two dilutions were inoculated by the pour-plate method. The cells were then left for incubation overnight at 37^{o}C. The colonies grown were counted and cfu/ml was counted and plotted. Survival rate was calculated by using the formula below:

Survival rate (%) = (log cfu N_1/log cfu N_0) x100%

(N_1 = Total number of the cells survived after each of the pH treatments, N_0 = Total number of alive cells before the treatment) [16].

Tolerance to gastric juice

To imitate gastric juice, 1x sterile PBS solutions, prepared at pH 2, pH 3, and pH4, were supplemented with fresh 3g /L pepsin. The isolate AB4 29 was incubated in each these solutions for 4h, and survival rate was assessed as before.

Tolerance to bovine bile acids

Four MRS liquid media, pH 6.8, each containing 0.5%, 1%, 1.5%, or 2% ox-bile, were prepared. The isolate AB4 29 was incubated in each these solutions for 3h, and survival rate was assessed as before.

Antibiotic resistance

AB4 29 strain was incubated overnight at 37^{o}C in MRS broth, pH 6.3. One millilitre of the overnight culture was then spread on MRS agar, pH 6.3. After waiting for 1 min, antibiotic discs containing kanamycin K 30, ampicillin AM 10, streptomycin S 10, tetracycline TE 30, gentamicin CN 30, chloramphenicol C 30, penicillin P 2 units, erythromycin E 15, rifampin RA 5, neomycin N 30, vancomycin VA 30, and control 00 were placed onto the plates, and allowed for overnight incubation at 37^{o}C. Following morning, antibiotics sensitivity was determined by measuring the diameters of the clear zones, formed around the discs, and the measured values were plotted [17].

Tolerance to sodium salts

The AB4 29 isolate was grown in MRS agar overnight at 37^{o}C. Sodium salts, 0.005 g/ml, (sodiumglycocholate hydrate, sodium taurodeoxycholate, sodium taurocholic acid) were prepared in MRS agar, pH 6.3. The colonies formed were immersed in the sodium salt solution and left for overnight incubation at 37^{o}C. Salt break-down by AB4 29 was checked and the results were recorded [18].

Cell surface hydrophobicity test

AB4 29 was grown overnight at 37^{o}C in MRS broth (pH 6.3). The culture was divided into four aliquots in sterile tubes. Cells were precipitated and 10 ml of phosphate-urea magnesium sulphate were added onto each of the cell pellets. This last step was performed twice. The

initial cell densities were set to 1 at 450nm and then 0.6 ml of *n*-hexadecane, *n*-hexane, xylene were added onto the cell suspensions (3ml). The final samples were then incubated for 15 min at 37^{o}C by gentle mixing every 2 min. The samples were kept at room temperature until a hydrocarbon layer formed. This step took approximately 25 min. The aqueous phase was removed and the bacterial density of the remaining mixture was measured. The hydrophobicity percentage was calculated by using the formula below.

Hydrophobicity % = (OD_{450nm} N0-OD_{450nm} N1) / OD_{450nm} x100%

(OD_{450nm} N_1 = absorbance value of the species after treatment with bovine bile acids, N_0 = absorbance value before application) [19].

Result and Discussion

Characterization of isolates

Morphological and partial biochemical characterization showed that all of the isolates were Gram-positive, catalase-negative and non-spore forming rod-shaped bacteria (data not shown). A phylogenetic analysis based on 16S ribosomal RNA (rRNA) gene sequence comparison showed that the isolates belonged to *Lactobacillus fermentum*. The sequence was submitted to GenBank (Accession Number: KJ865403).

Resistance to low pH, gastric juice, bile acids and sodium salts

AB4 29 strain produced a resistance curve similar to that of the control growth curves at pH 3 and 4. Small differences can be evaluated within the experimental error limits. Sensitivity started at pH points below 3. There was a 1000-fold decrease in the number of live cells at pH 2 and after two hour of incubation, beyond this point of time the number of the cells appeared to be stabilised (Figure 1).

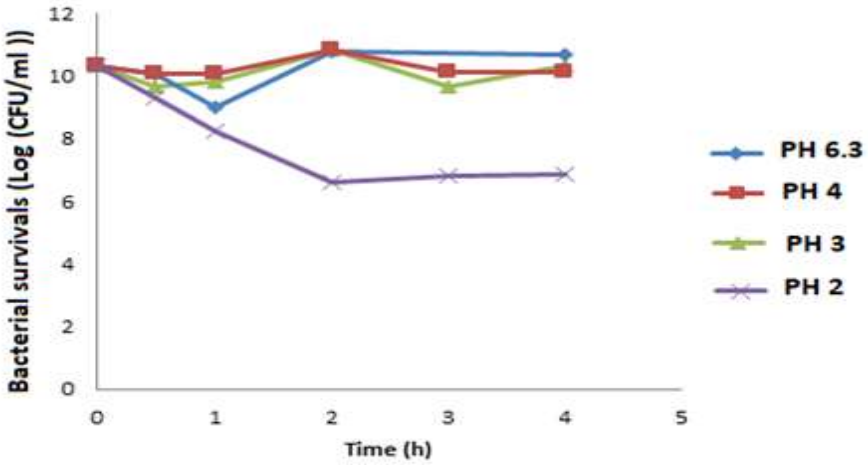

Fig 1 The number of live cells at low pH

AB4 29 was incubated at three different pH in phosphate buffered salt solution (PBS), containing pepsin. The cell number decreased approximately 100 times at pH 3 and 4, and approximately 10 billion times at pH 2 (Figure 2).

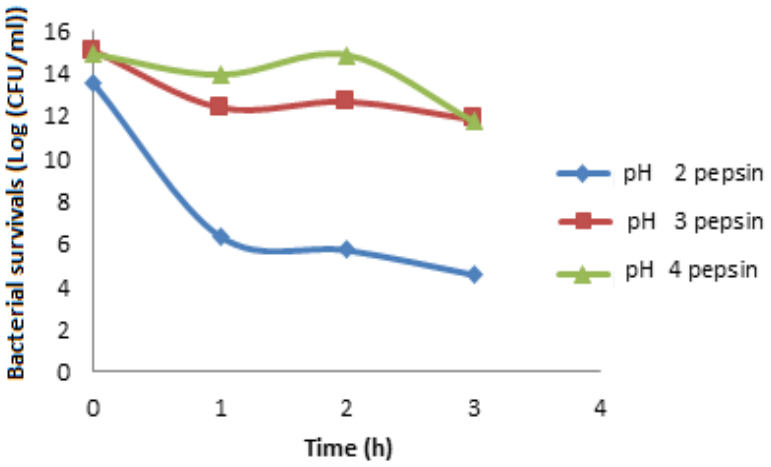

Fig 2 Survival rates of AB4 29

AB4 29 displayed the lowest resistance in this experiment. As can be seen (Figure 3), the number of cells decreased between 100 million to 1 trillion times.

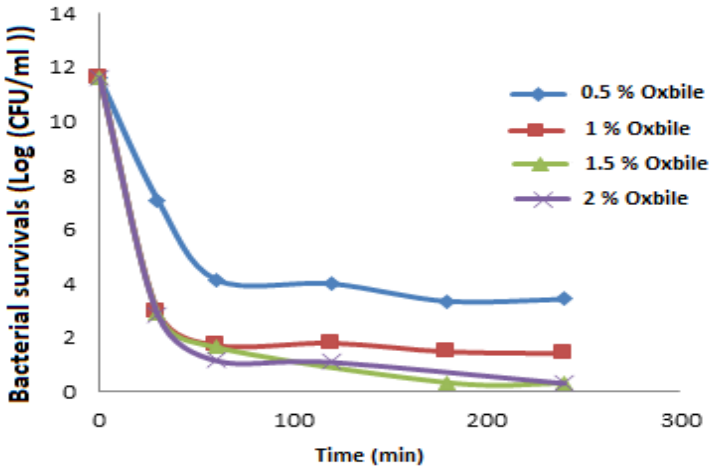

Fig 3 Tolerance to bile salt

The detoxification of sodium salts was determined qualitatively by means of salt zones formed around the colony. Enzymatic separation of the taurine portion from the deoxycholate enables the formation of zones around the colonies. Strain AB4 29 appeared to be capable of performing this reaction (Table 1).

Table 1 Break down of sodium salts

Bacteria	Sodium glycocholate hydrate	Sodium torodeoxycholate	Sodium torocolic acid	Sodium toroglycolate
AB4 29	+	+	+	-

High acid and bile tolerance of a probiotic candidate is very much desired because human body appears to have combined almost all the hostile environments within the confines of gastro-intestinal system. On one end, stomach pours in daily around 3 litres of strong acid, and the liver produces approximately 1 litre of bile salts, on the other end [20]. It appears that hydrolases seem to render the bacteria to be tolerant against bile salts [21,22]. It has been known that LABs could generally show significant resistance to the bile salts, between 0.3% and 0.4% concentrations at pH 7 [23]. This tolerance has seemed to decrease at pH points below 7 [24]. In some studies, it has been reported that much lower bile salt concentrations

could inhibit the growth of bacteria [25] and 0.3% could be the critical concentration [23]. In one of the reports it has been argued that toleration to bile salts can even be strain-dependent, that is it varied among the members of the same species [26]. In this study it was obvious that much higher bile salt concentrations, than those of the literature, were employed (Fig.3). Low tolerance could be a reflection of this approach. The acid tolerance profile of the strain AB4 29 at pH 2 (Fig. 1) was similar to those found in the literature, and this performance is likely to be improved further by adding glucose to the medium [27].

In the literature the maximum incubation time used was 100 min. In this study 5h of incubation was performed and after two hours the survival rate did not change and the number of live cells were stable. This finding might suggest that the protons of the medium were somehow titrated by the cellular metabolism, as it seems unlikely for the surviving cells to adapt to low pH in such a short time.

The survival rate obtained after the treatment with pepsin in an acid medium was also very similar to the that obtained in the absence of pepsin. The results in the literature are usually expressed in % rather than graphics, and the rates range from 60% to 98%. In this study, it is possible to say that the negative effect of pepsin on cells is very low. On the other hand, it is clear from the graphs that AB4 29 is more sensitive to trypsin.

Bacterial adhesion to hydrocarbons as a screening method for probiotic bacteria gives an insight into the role of hydrophobic interactions in adhesion [28]. Cell surface hydrophobicity of some strains of Lactobacillus has been found to be as high as 95% [29]. In some of the other studies relatively lower percentages have been mentioned [30]. AB4 29 appeared to have a rather weak surface hydrophobicity, ranging from 11 to 17% (Table 2), compared to those found in the literature. This issue can be clarified by more precise and non-controversial methods with devices measuring cell surface charge.

Table 2 Results of surface hydrophobicity

Solvent	**Hydrophobicity %**
n-Hexadecane	11,69
n-Hexane	17,62
Xylene	23,17
H_2O	17,05

To sum up, this initial study with a very limited project budget, suggests that LAB of infants fed with breast milk may be a good source of probiotic bacteria.

Antibiotic susceptibility

The sensitivity of AB4 29 to antibiotics was assessed qualitatively by using 11 different antibiotics (Figure 4).

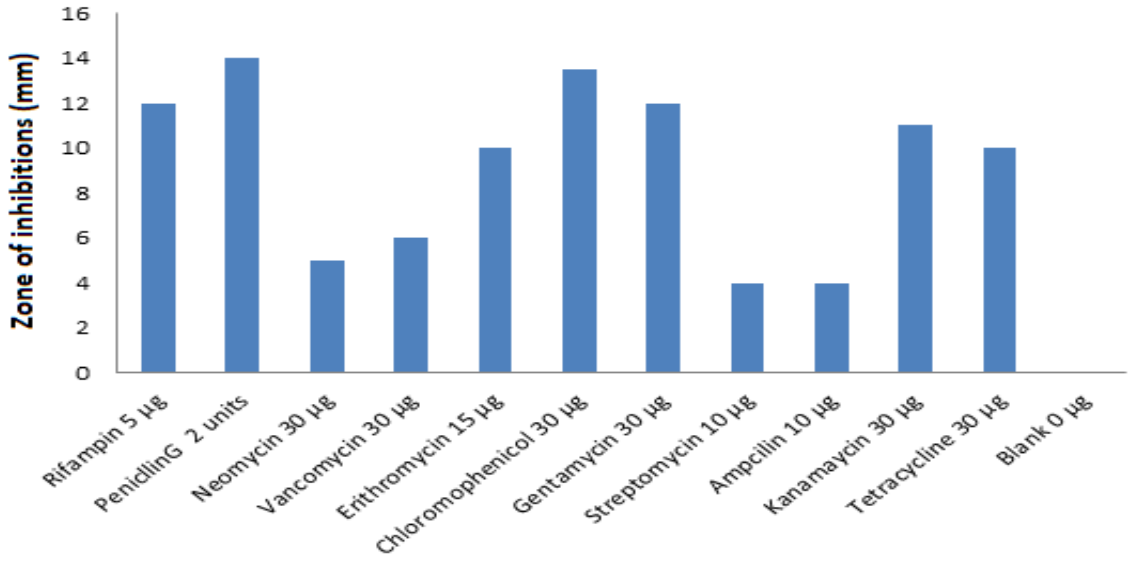

Fig 4 Antibiotic susceptibility of AB4 29

Surface hydrophobicity

Surface hydrophobicity was assessed by spectrophotometric measurement of cell turbidity in three organic solvents (Table 2). Compared with water, it was found that the rate of adhesion to *n*-hexadecane was lower than to that of water. The highest adhesion was obtained with xylene.

Conclusion

The study was performed with a project with modest budget. It enabled us to study some of the desired probiotic properties of the AB4 29 isolate of *Lactobacillus fermentum.* The experiment aiming at the investigation of the bile tolerance should be repeated by using the

much lower concentration figures indicated in the literature. Other findings were comparable to those found by other studies.

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