Internet Journal of Food Safety, Vol.12, 2010, p.187-191

Internet Journal of Food Safety

Evaluation of the Probiotic Properties and Antibiotic Resistance of Lactic Acid Bacteria Isolated from Awaze, Qotchqotcha and Tef dough, traditional Ethiopian fermented foods

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Abstract

Lactic acid bacteria were enumerated and isolated from traditional fermented products, awaze, qotchqotcha and tef dough, and their in vitro probiotic property was evaluated. Counts of lactic acid bacteria isolated from the different fermented products ranged between 10^5cfu/g – 10^9 cfu/g. Significant difference was observed between and among the samples ($p < 0.05$). Based on their phenotypic characters the isolates were grouped as *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc*. Antibiotic resistance of the isolates against penicillin, vancomycin, tetracycline, ampicillin, kanamycin, clindamycin and gentamicin ranged between 14% and 43%. Variability in the pattern of drug sensitivity was also observed among the isolates. About 58% of the tested isolates survived bile concentration greater than 0.3 % and 57% and 47% survived pH 3 for three and six hours, respectively. Under all pH condition further incubation reduced the number of lactic acid bacteria used during the experiment. Some of the isolates could survive in the gastric and intestinal environments and, thus have probiotic potential.

Key words: LAB, probiotics, drug resistance, acid tolerance, bile tolerance

Introduction

Intestinal microflora is composed of a wide diversity of bacteria that can perform important functions (Salminen *et al.*, 1995). Lactic acid bacteria, which are found commonly as resident microflora of the gastro-intestinal and genitor-urinary tract of vertebrates (Carr, 2002), are considered as the major probiotic organisms (Collins *et al*., 1998). Probiotics has been defined as "non-pathogenic microorganisms that, when ingested in certain numbers, exert a positive influence on the host physiology and health beyond inherent general nutrition" (Ouwehand *et al*., 2002).

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Strains of *Lactobacillus*, such as *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus casei* and *Lactobacillus gasseri* make a significant portion of probiotic products. Mechanisms of action of probiotics include competitive exclusion of pathogen binding, production of antimicrobial substances and competition for nutrients (Parvez *et al*., 2006).

Several reports have indicated that probiotic lactic acid bacteria are capable of inhibiting pathogenic microorganisms. Some of the probiotic lactobacilli posses inhibitory activity against the growth of pathogenic microorganisms such as *Salmonella*, *Escherichia coli* (Drago *et al*., 1997), *Listeria monocytogens* (Harris *et al.*, 1989), *Shigella*, *Pseudomonas* and *Helicobacter* (Servin, 2004). Probiotic lactic acid bacteria have also been reported

to reduce urinary tract infection, bacterial vaginosis and yeast vaginitis (Reid *et al*. 1995). Lactic acid bacteria improve lactose digestion and eliminate symptoms of lactose intolerance (de Vrese, *et al*., 2001). Some lactic acid bacteria belonging to *Lactococcus* and *Bifidobactrium* spp. are reported to metabolize cholesterol (Klaver and van der Meer, 1993). Probiotic lactic acid bacteria also play role in immune modulation in humans (Zoumpopoulou *et al.*, 2008).

Although a number of studies demonstrated the inhibitory effect of lactic acid bacteria, isolated from traditional Ethiopian foods, against some food borne pathogens, Bacha et al (2009) and Tesfaye et a. (2010) recently evaluated the in vitro probiotic properties of lactic acid bacteria isolated from various Ethiopian traditional fermented foods and beverages. Most of the traditional fermented products of Ethiopia are consumed without further heating or any other form of processing. Thus they are ideal to carry probiotic bacteria into the digestive system. The objective of this study was to assess the in vitro probiotic potential of lactic acid bacteria isolated from two traditional fermented condiments, Awaze and Qotchqotcha. and fermented Tef dough to be baked into Enjerria,a traditional pan cake. The drug resistance pattern of the isolates was also determined. Drug resistance in probiotic organisms may warrant the establishment of these organisms in the intestinal walls for longer periods and guarantee their continual probiotic role. However the risk of transferable drug resistance to food borne pathogens may raise a public health concern.

Material and Methods

Microbiological analysis. A total of 30 samples comprising 10 each of Awaze, Qotchqotcha and tef dough were aseptically collected for this study. Awaze and Qotchqotcha samples were purchased from 10 different supermarkets and ready-to-bake Tef dough samples were collected from 10 different households in Addis Ababa. Samples were analyzed with in 3 – 6 hours of arrival at the laboratory.

A 25 g sample of each sample was placed aseptically in a sterile stomacher bag and homogenized in 225 ml of sterile 0.1% (w/v) peptone water using a Stomacher lab blender (model 400, Seward JAC, London). Serial ten-fold dilutions were prepared and, from appropriate dilutions, 0.1 ml aliquots were spread plated in duplicate on pre-dried surfaces of MRS (de-Mann, Rogosa and Sharp) agar (Oxoid) plates. The plates were incubated under anaerobic condition, using anaerobic jar (BBL, Gas Pak Anaerobic Systems) at 30 to 32℃ for 48 h. All colonies were counted as lactic acid bacteria.

After colony counting, 10 to 15 colonies were randomly picked from countable MRS agar plates for further differentiation. Colonies of lactic acid bacteria were transferred into 5 ml BHI (brain heart infusion) broth (Oxoid) and purified by repeated streaking on MRS agar. The pure cultures of lactic acid bacteria were then characterized based on cell morphology, Gram reaction (Gregerson, 1978), Catalase test and Cytochrome Oxidase test (Kovacs, 1956). Production of gas from 5% glucose was detected in MRS broth with inverted Durham tube after incubation at 32 ^{0}C for upto five days.

Drug sensitivity testing. The isolates were tested for their susceptibility on Muller Hinton Agar by the standard disc diffusion technique (Jorgenson *et al*., 1999) with 8 Oxoid drug discs: gentamycin (Gen), (10µg); clindamycin (Cli), (2µg); ampicillin (Amp), (10µg); tetracycline (Tet), (30µg); vancomycin (Van), (30µg); streptomycin (Str), (10µg); penicillin (Pen), (10µg); and Kanamycin (Kan), (30µg). Plates were incubated at 32^{o}C for 18-20 hours. Diameter of zones of inhibition was measured in mm and interpreted as susceptible (S), or resistant (R) (Jorgenson *et al*., 1999). All intermediate results were considered sensitive for the purpose of interpretation.

In-vitro Probiotic Evaluation of LAB. A total of 257 isolates were used for this study. Samples of overnight cultures (20 µl corresponding to 6 log cfu/ml) were spotted on to pre-dried surfaces of MRS agar plates supplemented with bile salt (0.1 to 1% w/v oxgall at 0.1 intervals) (Sigma Chemical Co. St Louis, Missouri, USA) according to Hyronimus *et al*. (2000). Plates were incubated for 5 days. The minimal inhibitory concentration (MIC) of bile for a strain was determined as the lowest concentration totally inhibiting the growth of spots as judged from visual examination of spots. Culture-free BHI broth plated on MRS agar plate was used as a control. Isolates which were able to withstand concentrations over 0.3% were considered for further analysis.

Acid tolerance was determined for isolates that survived bile concentration greater than 0.3% (Hyronimus *et al*., 2000). Cultures were grown in BHI broth at 37^{0}C overnight and 1 ml was separately sub-cultured in 10 ml of fresh MRS broth adjusted to pH values of 2, 2.5, or 3.0 using hydrochloric acid (3.0 M) to simulate the gastric environment. The initial inoculum level was determined by plate counting on MRS agar. Samples were incubated for 3 and 6 hours at 37^{0}C. After appropriate incubation, 1 ml of the culture was diluted in pre-sterilized 9 ml phosphate buffer (Sigma, St. Louis, USA) to neutralize the medium acidity and similarly plated for counting. Culture-free BHI broth media of pH values of 2.0, 2.5, or 3.0 were used as control. Viable cell count was made after 24 to 48 h of incubation under anaerobic condition using anaerobic jar (BBL, Gas Pack System). The survival rate was calculated as the percentage of LAB colonies grown on MRS agar compared to the initial bacterial concentration.

Results

The isolates were grouped to different genera as indicated in (Table 1). Based on their glucose fermentation profile, a total of 249 isolates were grouped as homofermentative and 16 were heterofermentative. A total of 257 isolates were obtained from the fermented products at varying frequencies (Table 2).

Table 1. Distribution of the different genera among the fermented products

Food item	*Lacto bacillus*	*Leuco nostoc*	*Pedio coccus*	*Lacto coccus*
Awaze	52	1	27	7
Qotchqotcha	69	3	22	6
Tef dough	58	0	11	1

Table 2. Criteria for grouping of lactic acid bacteria to different genera.

Colony characteristics	Shape	Glucose fermentation		LAB genera
		Homo[1]	Hetero[2]	
White / round	Rod	+	+	*Lacto bacillus*
White / round	Cocci in tetrad	+	-	*Pedio coccus*
White/ round	Cocci in pairs or chains	+	-	*Lacto coccus*
White / round	Cocci in pairs or chains	-	+	*Leuco nostoc*

[1]HomoFermentative; [2]Heterofermentative

The count of lactic acid bacteria in the fermented products ranged between 5 log cfu/g and 9 cfu/g The highest count was noted for an awaze sample (9.8 log cfu/g) and the smallest count was for qotchqotcha (log 5.3/g) (data not given). Significant difference was observed between and among the samples ($p < 0.05$).From the tested antibiotics the most frequent resistance was to penicillin 43%, followed by vancomycin 41% (Table 3). A total of 55 multiple drug resistance (MDR) patterns were observed among the lactic acid bacteria isolates. Three lactobacilli (qotchqotcha isolates) were resistant to seven antibiotics. The most frequent resistance pattern was to two antibiotics Pen/Amp, followed by resistance to five antibiotics (Tet/Kan/Cli/Str/Gen) (Table 4). A total of 257 isolates of LAB comprising *Lactobacillus* (177), *Lactococcus* (13), *Pediococcus* (61) and *Leuconostoc* (6) were tested for bile tolerance (Table 5). Only 36% of the isolates could survive in bile concentration of $\geq$ 1%. Among these, *Lactobacillus* spp. were the dominant ones. Isolates obtained from the more acidic fermented products (Awaze and qotchqotcha) could survive at higher bile concentrations than those isolated from teff dough.

Only *Lactobacillus* isolates could survive at pH 2 for three hours. *Lactococus* and *Pediococcus* isolates could survive only at pH 2.5 or pH 3 (Table 6).

Table.3 Antibiotic resistance patterns of lactic acid bacteria isolated from fermented products

Isolates	No.	Number of resistant isolates							
		Gen	Cli	Amp	Tet	Van	Str	Pen	Kan
Lacto bacilli	132	10	22	64	43	62	30	81	22
Lacto cocci	14	1	6	0	2	6	4	1	3
Pedio cocci	49	16	30	5	26	13	22	3	34
Leuco nostoc	5	1	2	1	1	1	2	1	2
Total	200	28	60	70	72	82	58	86	61

Table 4. Most frequent multiple drug resistance pattern in LAB.

No. of drugs	**MDR Pattern**	**No. of resistant isolates**
7	Pen/Van/ Tet /Amp/Kan/ Cli /Str	3
6	Pen/Van/ Tet /Amp/Kan/ Str	4
5	Tet / Kan / Cli / Str / Gen	14
4	Tet / Kan / Cli / Str Pen / Van / Tet / Amp	5 6
3	Amp / Kan / Str Pen / Van / Str Pen / Van / Tet Van/ Tet /Kan	4 4 4 3
2	Van/ Cli Pen/ Van Van/ Tet Kan/ Cli Pen/ Amp	3 10 7 6 22

Where: Gen, gentamycin; Cli, clindamycin; Amp, ampicillin; Tet, tetracycline; Van, vancomycin; Str, streptomycin; Pen, penicillin; Kan, Kanamycin and MDR, multiple drug resistane.

Table 5. Determination of minimal inhibitory concentration of bile salt for lactic acid bacteria isolated form tef dough, awaze and qotchqotcha

Isolates	Total no of isolates	Bile concentration and number of resistant isolates														
		0.1%			0.3%			0.5%			1%			>1%		
		Aw	Qo	TD	Aw	Qo	TD	Aw	Qo	TD	Aw	Qo	TD	Aw	Qo	TD
Lactobacilli	177	-	3	12	6	23	28	7	15	11	4	8	6	35	19	-
Lactococci	13	-	-	1	-	6	-	1	1	1	2	-	-	1	-	-
Pediococci	61	2	-	-	10	10	6	6	7	3	4	2	2	6	3	-
Leuconostoc	6	-	-	-	-	2	-	2	1	-	-	-	-	1	-	-
Total Isolates	257	2	3	13	16	41	34	16	24	15	10	10	8	43	22	-

Aw, Awaze; Qo, Qotchqotcha; TD, Tef Dough

Table 6. Survival of isolates at different pH values

Isolates	Survival (%)					
	pH 3.0		pH 2.5		pH 2.0	
	3h	6h	3h	6h	3h	6h
Lactobacillus	48	38	24	14	10	-
Lactococcus	67	67	-	-	-	-
Pediococcus	83	67	17	-	-	-

Discussion

Significant difference was observed ($p < 0.05$) in count of lactic acid bacteria between and within samples. In the fermented products, lactic acid bacteria initiated the fermentation process at a level of 4 log cfu/g and reached about 9 log cfu/g at the end of fermentation period as observed in Idris *et al.* (2001). However, contrary to the observations of Idris *et al.* (2001), most of the lactic acid bacteria isolated from awaze fermentation were homofermentative. As the fermentation was a spontaneous process and no particular starter culture was used, variations in bacterial type are possible.

Lactic acid bacteria isolates showed varying degree of susceptibility to the antibiotic tested. Most of our lactic acid bacteria were resistant to vancomycin, as report by Danielson and Wind (2003). On the other hand, although lactobacilli are usually sensitive to antibiotic that inhibit cell wall synthesis, such as penicillin and ampicillin (Danielson and Wind, 2003), our lactobacilli isolates were resistant to penicillin. Isolates from tef dough qotchqotcha and awaze showed variability in their pattern of resistance to the antibiotics used in this study. This variability could be due to inherent differences among the isolates. Antibiotic resistance may be desirable in a probiotic organism as it can survive in the gut environment despite exposure to antibiotics during treatment. However, the possibility of horizontal transfer of drug resistance to pathogens in the gut environment is a point of concern.

Varying degree of survival at different pH values was observed among and between the different genera of lactic acid bacteria. Although most isolates could survive at pH 3 for the first 3 hours, further incubation for three more hours decreased the number of survivors. Similar observation was also made by Hyronimus *et al.* (2000. Unlike some of our *Lactobacillus* isolates which survived in pH 2, all their isolates did not survive pH 2.5 (Hyronimus *et al*,.2000). In our study, only *Lactobacillus* isolates could survive in the gastric environment for three hours which is the duration food is supposed to stay in the stomach. This indicated their potential to pass to the intestine where they may function as possible probiotic microorganisms.

A study conducted by Succi *et al*., (2005), indicated that the lactic bacteria isolated from cheese showed a very good tolerance to bile concentration. Similar condition was also observed for lactic acid bacteria isolated from our fermented products. Survival of the lactic acid bacteria isolates in the presence of bile salt could be attributed to the presence of specific genes that code for bile salt hydrolase enzyme (Gilliland *et al*., 1985; Tanaka *et al*, 1999) that is responsible for the detoxification of the bile salt by converting it to its simpler components.

This study showed that most of the lactic acid isolates from the fermented products can survive the high acidity of the gastric environment and bile in the intestine. Although this may indicate their potential for probiosis, their ability to adhere to the intestinal epithelial cells and to produce antimicrobial substances should be evaluated to use them as probiotic starter cultures.

Acknowledgements

AD acknowledges the financial support of Sida/SAREC obtained through the Graduate Program, Addis Ababa University.

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