Assessment of the antimicrobial activity of lactic acid bacteria isolated from Borde and Shamita, traditional Ethiopian fermented beverages, on some food-borne pathogens and effect of growth medium on the inhibitory activity.

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Abstract

The antimicrobial activity of lactic acid bacteria (LAB) isolated from fermented products on various food borne pathogens is well documented. This prompted the study to evaluate the *in-vitro* antimicrobial activity of LAB isolated from two traditional Ethiopian fermented beverages on *Staphylococcus aureus, Shigella flexneri, Salmonella* spp*. and Esherichia coli* O157:H7. *Lactobacillus* isolates were separately grown in MRS and LAPTg broths and their antimicrobial activity was tested against the test strains using the disc diffusion method. All isolates, except *Esherichia coli* O157:H7, showed additional 3 to 4 mm of inhibition zone over the control. This was <3 mm for *Esherichia coli* O157:H7. *Lactobacillus* isolates were the most inhibitory to the test strains followed by *Pediococcus, Streptococcus* and *Leuconstoc* isolates in that order. Among the test strains, the most sensitive were *Shigella flexneri* for *Lactobacillus* and *Leuconostoc* isolates, *Salmonella* for *Streptococcus* isolates and *Staphylococcus aureus* for *Pediococcus* isolates. *E. coli* O157:H7 was the least sensitive in all cases. Study on the effect of growth media for *Lactobacillus* on its antimicrobial activity showed that inhibition was significantly greater on *Staphylococcus aureus* and *Esherichia coli* O157:H7 test strains when *Lactobacillus* was grown on LAPTg broth than in MRS broth. The study sowed that LAB involved in the fermentation of traditional beverages had an antimicrobial property against various food borne pathogens and the inhibitory products were extracellular and diffusible. The observed inhibitory property of LAB was influenced by the medium they grew in.

Key words: Lactic acid bacteria, inhibition, *E. coli* O157:H7, *Salmonella, Shigella flexneri, Staphylococcus aureus,* traditional fermented beverages

Introduction

Different reports show that most lactic acid bacteria (LAB) produce substances that inhibit pathogenic, non-pathogenic and spoilage organisms in fermenting foods and beverages (Gilliland & Speck, 1975; Schillinger & Lucke, 1989). Lactic acid bacteria have been used successfully, with few adverse effects, to prevent antibiotic associated diarrhea, to treat acute infantile diarrhea and recurrent *Clostridium difficile* disease and to treat various diarrheal illnesses (Siitonen, Vapaatalo, Salminen, Gordin, Saxelin, Wikberg, & Kirkkola, 1990; Saavedra, Bauman, Oung, Perman, & Yolken, 1994; Biller, Katz, Floves, Buie, & Gorbach, 1995).

The antagonistic property is attributed to the lowered pH, the undissociated acids and production of other primary and secondary antimicrobial metabolites produced by LAB. The metabolites produced by the fermentation process, except the volatile ones, are kept in the foods and result in growth inhibition of food spoilage or poisoning bacteria and detoxification of noxious compounds of plant origin, (Gilliland & Speck, 1975; Schillinger & Lucke, 1989; Salminen, Isolauri & Salminen, 1996). In the case of traditional foods and beverages fermented by LAB, the fact that the products arrest the survival and growth of pathogens is more related to the *in situ* action of lactic acid bacteria and their metabolites as observed in fermented cereal gruels and fermented milk (Svanberg, Siogren, Lorri, Svennerholm & Kaijser, 1992; Ashenafi, 1993; Kingamkono, Sjorgen, Svanberg & Kaijser, 1995.), and in the fermentations of kocho and tef dough (Nigatu and Gashe, 1994 a,b) siljo (Dessie, Abegaz, & Ashenafi, 1997) and awaze and data (Idris, Mehari, & Ashenafi,, 2001).

Borde and Shamita are important traditional fermented beverages in Ethiopia. They are produced by an over-night fermentation of certain cereals predominantly by LAB. They are low-alcohol products and are consumed in large amounts as meal replacements. The production, microbial dynamics and the chemical changes occurring during the fermentation of Borde and Shamita have been studied by Ashenafi & Mehari (1995), Bacha, Mehari & Ashenafi (1998, 1999) and Abegaz, Beyene, Langsrud & Narvhus,. (2002a,b).

The aim of this study is, therefore, to evaluate the *in-vitro* antimicrobial activities of lactic acid bacteria isolated from Borde and Shamita, traditional Ethiopian fermented

beverages, on the growth and survival of some food borne pathogenic microorganisms.

Materials and methods.

Sample collection

Samples of Borde and Shamita (250ml/brewer) were separately collected from ten *Borde* and Shamita household brewers in Addis Ababa using sterilized flasks and brought to the laboratory for isolation of lactic acid bacteria. The samples were kept for 2-4h in the refrigerator until analysis was conducted.

Isolation and grouping of lactic acid bacteria

For the isolation of lactic acid bacteria, a volume of 0.1 ml of appropriate dilutions of borde and Shamita was plated on MRS (OXOID) agar plates. Inoculated plates were incubated anaerobically at 32^{o} C for 48 h in an anaerobic jar (BBL). Ten to twenty colonies were randomly picked from countable MRS agar plates, purified by repeated plating and studied for their Gram reaction, cell and colony morphology, and catalase reaction. Gram-positive, catalase-negative, cocci or rod-shaped isolates with characteristic cell arrangements were considered as lactic acid bacteria. Further grouping in to different genera was made by testing for gas production in MRS broth containing 5% glucose. Gas production was detected in inverted Durham tubes after incubation at 32 ^{0}C.

Test strains

Salmonella sp., *Shigella flexneri*, *Staphylococcus aureus* and *E. coli* O157:H7 were used as test strains to evaluate the antimicrobial effects of LAB isolated from Borde and Shamita. *Salmonella* sp., *Shigella flexneri* and *Staphylococcus aureus* were clinical isolates obtained from the Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa. *E. coli* O157:H7 was isolated from hamburger meat and was obtained from the Food Microbiology Laboratory of Howard University, USA .

Determination of antimicrobial activity of LAB in broth culture

LAB isolates were separately grown in test tubes without agitation in 10 ml MRS broth (OXOID) and LAPTg broth (Raibaud, Caulet, Galpin & Mocquot, 1961) at 32^{o}C for 48 hours. LAPTg broth contained (g/l distilled water): yeast extract, 10; peptone, 15; tryptone, 10; glucose, 10; Tween 80.

The antimicrobial activity of the LAB isolates was determined by modifying the disc diffusion method of Hamadan & Mikolajcik (1974) and Apella, Gonzalez, Nader de Macias, Romero & Oliver (1992). Sterile filter discs (12mm) were dipped in to the culture broth of lactic acid bacteria incubated for 42 hours and placed on solidified Muller-Hinton agar (OXOID) seeded with 12 to 14h cultures of test microorganisms. The plates were kept at 4^{o}C for 3 to 4 h to permit diffusion on the assay material, and incubated at 37^{o}C for 14 to 16h. Discs dipped in un-inoculated MRS broth served as control. Zones of inhibition were then measured. The antibiotic activity tests were done in duplicates and the mean values were recorded.

Results

A total of 118 LAB were isolated from ready to consume Borde and Shamita and tentatively grouped in to *Lactobacillus* sp. (20 homofermentors and 40 heterofermentors), *Leuconostoc* sp. (15), *Pediococcus* sp. (18) and *Streptococcus* Sp. (25).

Lactobacillus isolates resulted in the highest diameter of inhibition zone than the other LAB isolates on the test strains (Table 1). Mean differences in inhibition zone diameters between broth where *Lactobacillus* isolates grew and the control was >4 mm for *Salmonella* spp, *Shigella flexneri* and *Staphylococcus aureus*, whereas it was only 2.76 mm for *E. coli* O157:H7. *Shigella flexneri* was the most sensitive to *Lactobacillus* isolates followed by *Salmonella* spp., *Staphylococcus aureus* and *E. coli* O157:H7 in that order.

Table 1. Diameter of zones of inhibition (mm) produced by *Lactobacillus* isolates on the test strains as assessed by the disc diffusion method.

Lactobacillus isolate #	Test strains			
	Salmonella spp	*Shigella flexneri*	*Staph. aureus*	*E.coli* O157:H7
9	17.3	18.0	16.2	16.2
25	18.0	17.2	17.0	15.2
69	16.7	17.6	17.8	16.0
70	17.2	17.2	17.4	15.3
83	17.3	17.0	17.0	16.1
Mean ± SD*	17.3±0.46	17.4±0.4	17.08±0.4	15.76±0.47
%CV**	2.7	2.3	2.3	2.9

Control	13.2	13.2	13.2	13.0

*SD, Standard deviation; **CV, Coefficient of Variation

Leuconostoc isolates also showed some inhibitory activities against the test strains (Table 2). Mean differences in inhibition zone diameters between broth where *Leuconostoc* isolates grew and the control was between 3.16 and 3.28 for *Salmonella* spp, *Shigella flexneri* and *Staphylococcus aureus.* This was only 2.14 for *E. coli* O157:H7. *Salmonella* spp, and *Shigella flexneri* were the most sensitive to *Leuconostoc* isolates.

Table 2. Diameter of zones of inhibition (mm) produced by *Leuconostoc* isolates on the test strains as assessed by the disc diffusion method

Leuconostoc isolate #	Test strains			
	Salmonella spp	*Shigella flexneri*	*Staph. aureus*	*E.coli* O157:H7
4	16.0	16.6	16.2	15.4
7	15.8	16.7	16.4	15.0
10	17.1	16.0	16.4	15.8
13	16.3	16.4	16.0	15.3
24	16.2	16.2	15.8	15.2
Mean ± SD*	16.28 ± 0.50	16.38±0.29	16.16±0.28	15.34±0.30
%CV**	3.1	1.8	1.6	1.9
Control	13.0	13.1	13.0	13.2

*SD, Standard deviation; **CV, Coefficient of Variation

Pediococcus isolates showed a relatively larger zone of inhibition on the test strains next to *Lactobacillus* isolates. Mean differences in inhibition zone diameters between broth where *Pediococcus* isolates grew and the control was between 3.2 and 3.5 for the test strains except for *E. coli* O157:H7 (Table 3). *Staphylococcus aureus* was the most sensitive to inhibitory activity of *Pediococcus* isolates.

Table 3. Diameter of zones of inhibition (mm) produced by *Pediococcus* isolates on the test strains as assessed by the disc diffusion method

Pediococcus isolate #	Test strains			
	Salmonella spp	*Shigella flexneri*	*Staph. aureus*	*E.coli* O157:H7
3	16.0	16.2	16.2	15.7
9	16.5	16.5	16.0	15.4
13	16.4	16.4	16.5	15.0
14	16.2	16.6	16.4	15.6
15	17.1	16.4	16.4	15.1
Mean ± SD*	16.44±0.42	16.42±0.15	16.3±0.2	15.36±0.31
%CV**	2.5	0.9	1.2	1.9
Control	13.2	13.0	12.8	13.0

*SD, Standard deviation; **CV, Coefficient of Variation

Streptococcus isolates also showed some degree of inhibition against the test strains and they were the most effective against *Salmonella* spp (Table 4).

Table 3. Diameter of zones of inhibition (mm) produced by *Pediococcus* isolates on the test strains as assessed by the disc diffusion method

Streptococcus isolate #	Test strains			
	Salmonella spp	*Shigella flexneri*	*Staph. aureus*	*E.coli* O157:H7
5	16.0	16.2	16.4	15.5
7	16.4	16.4	16.1	15.3
12	15.5	16.1	16.2	15.1
13	16.2	16.3	16.0	15.2
19	16.4	16.5	16.2	15.4
Mean ± SD*	16.1±0.37	16.3±0.16	16.18±0.15	15.3±0.16
%CV**	2.3	1.0	0.9	1.0
Control	12.5	13.0	13.0	13.

*SD, Standard deviation; **CV, Coefficient of Variation

In general, when inhibition zones produced by isolates belonging to each LAB genus were compared, differences were not significant ($p>0.05$). However significant difference was observed in degree of inhibition between the control and the LAB strains in all cases ($p<0.05$). Based on the differences in inhibition zones produced by LAB cultures and the controls, *Lactobacillus* isolates were the most inhibitory to the test strains followed by *Pediococcus, Streptococcus* and *Leuconstoc* isolates in that order. Among the test strains, the most sensitive were *Shigella flexneri* for *Lactobacillus* and *Leuconostoc* isolates, *Salmonella* for *Streptococcus* isolates and *Staphylococcus aureus* for *Pediococcus* isolates. *E. coli* O157:H7 was the least sensitive in all cases.

The study on comparison of inhibitory property of *Lactobacillus* isolates, when grown in two different types of broths, showed that there was no significant difference arising from growth media in case of inhibition on *Salmonella* isolates and *Shigella* flexneri ($P>0.05$). However, inhibition was significantly greater on *Staphylococcus aureus* and *E. coli* O157:H7 when *Lactobacillus* was grown in LAPTg broth than in MRS broth ($p<0.05$) (Table 5).

Table 5. Diameter of zones of inhibition (mm) produced by *Lactobacillus* isolates grown in LAPTg or MRS broth on the test strains as assessed by the disc diffusion method

Lactobacillus	*Salmonella* spp.		*Shigella flexneri*		*Staphylococcus aureus*		*E. coli* O157:H7	
Isolate #	LPTG broth	MRS broth	LPTG broth	MRS broth	LPTG broth	MRS broth	LPTG broth	MRS broth
9	19.3	17.3	17.4	18.0	18.1	16.2	17.0	16.2
25	19.4	18.0	17.6	17.2	18.7	17.0	16.6	15.2
69	18.0	16.7	17.8	17.6	17.4	17.8	16.6	16.0
70	16.9	17.2	17.1	17.2	18.0	17.4	17.0	15.3
83	17.3	17.3	17.0	17.4	18.3	17.0	16.5	16.1
Mean ± SD*	18.18±1.14	17.3±0.46	17.38±0.34	17.48±0.34	18.10±0.47	17.08±0.59	16.74±0.24	15.76±0.47
%CV**	6.27	2.66	1.95	1.95	2.51	2.45	1.43	2.98
Control	13.7	13.0	13.3	13.1	13.0	13.2	13.3	13.0

*SD, Standard deviation; **CV, Coefficient of Variation

Discussion

The culture filtrate from LAB, that included the genera of *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* sp., isolated from Borde and Shamita were tested by disc diffusion method to know if the antimicrobial metabolites produced by LAB were extracellular and released into the growth medium. In this test all the test strains were inhibited indicating that the inhibitory metabolites produced by the isolates were extracellular and diffusible because inhibition took place by diffusing through a layer of agar. All the LAB isolates belonging to the four genera inhibited the growth of the test strains to varying degrees. Similar to our findings, Kivanc (1990) observed varying degrees of inhibition of various food borne pathogens by cell-free filtrates of LAB. The *in vitro* study of Apella, Gonzalez, Nader de Macias, Romero & Oliver (1992) also showed the inhibitory activity of *Lactobacillus casei* and *Lactobacillus acidophilus* on the growth of *Shigella sonnei*.

In-vitro Inhibition of *E. coli* O157:H7 by LAB was observed in Trypticase Soy broth (Brashears, Reilly & Gilliland, 1998), in agar spot tests (Brashears, Jaroni & Trimble, 2003), and on agar plates (Caridi, 2002). Among the test bacteria used in this study, *E. coli* O157:H7 was relatively resistant to the antimicrobial activity of LAB than the other test strains. *E. coli* O157:H7 is more tolerant to some organic acids than many other infectious pathogens and can survive well in acidic food and beverages (Rochelle, Clavero, & Beuchat, 1996). There are also reports that indicate some microorganisms produce an acid-tolerance response system that protects them against severe acid stress for longer periods (Foster & Hall, 1991). This may be the reason for resistance of *E. coli* test strain when compared to the other test strains.

As our LAB were grown in broth media containing glucose, the observed inhibition might arise from the acid produced. Varadaraj, Devi, Keshava & Manjrekar (1993) observed moderate inhibition of some food borne pathogens and other bacterial species by neutralized culture filtrates of LAB using a well diffusion assay. McLean & McGroarty (1996) also showed that about 60% of the antimicrobial activity of culture filtrates of LAB was removed when the filtrates were neutralized to pH 6.5 with NaOH.

The diameter of zone of inhibition from the culture filtrate of *Lactobacillus* isolates, grown in LAPTg broth, on some of the test strains was significantly higher than that from culture filtrate of *Lactobacillus* isolates grown in MRS broth. This difference might be due to the difference in the constituent of LAPTg and MRS broths. Although LAPTg broth is more of a general-purpose medium which is nutritionally much more inferior to MRS broth, it was interesting to note that it conferred stronger antimicrobial property to *Lactobacillus* grown in it. This indicated that the media composition affects the production of antimicrobial metabolites produced by *Lactobacillus* isolates. A similar result was reported by Apella, Gonzalez, Nader de Macias, Romero & Oliver (1992) where the media type in which the LAB were grown affected production of antimicrobial metabolites by LAB.

MRS broth contained more glucose (20%) than LAPTg broth (10%) and, thus, more acid would be expected to be produced by *Lactobacillus* isolates in MRS broth. The observed higher inhibition from growth of *Lactobacillus* isolates in LAPTg may not, therefore, be accounted for only in terms of acid production. This finding suggested that LAB might produce more metabolites with antimicrobial property when grown in a less nutritious medium.

In conclusion, LAB involved in Borde and Shamita fermentation had an antimicrobial property against various food borne pathogens. The inhibitory products are extracellular and diffusible. The medium where LAB grew could have a contribution to the inhibition of some food-borne pathogens introduced into it.

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