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ANTAGONISM AND PRIMARY *in vitro* PROBIOTIC EVALUATION OF LACTIC ACID BACTERIA (LAB) RECOVERED FROM *Ergo*

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ABSTRACT

Ergo is a naturally processed indigenous fermented dairy product, which is commonly prepared at household level and consumed in Ethiopia. The fermentation of this product is lactic acid bacterial dominated process. During this study 500 lactic acid bacterial isolates were recovered from *ergo* and tested for antagonism against four foodborne pathogens (*E. coli* O57:H7, *Salmonella typhimurium* DT104, *Shigella shigelea* SH10-1 and *Staphylococcus aureus* MERSA). Based on screening agar spot test method, 41.8% of LAB formed lysis zone against the test foodborne pathogens on modified MRS agar medium. Crude extracts of 40 lactic acid bacteria (LAB) that inhibited the test pathogens with ≥ 10 mm lysis zone were tested against the pathogens. Of these 40 LABS, 55% (22 LAB isolates) of extracts were observed inhibitory to the test pathogens with the well diffusion method. Purified extracts (ammonium sulfate precipitated) of 72% (16 isolates) of those shown active with crude extracts test were exhibited inhibitory activity against the test foodborne pathogens. From inhibitory LAB of purified extracts, 17.5% were found antagonistic to three or four of the test foodborne pathogens. The same 16 LAB isolates with antagonistic characteristics were tested for acid tolerance at pH 2.0, 2.5 and 3.0 for 3 and 6 hrs. Of these 16, none of the LAB isolates survived exposure to pH 2.0 for 3 hrs. Only 7 LAB isolates survived (with survival rate of >50%) the exposure at pH 2.5 for 3 and further incubation for 6 hours. All 16 isolates tolerated pH 3.0 for 3 hours. Further incubation for 6 hours reduced the survivors to 12. The 7 LAB isolates those survived exposure to pH 2.5 for 6 hrs were tolerated 0.3% bile (with survival rate of >95%) for 48 hours. The study indicated that the LAB isolates that are observed antagonistic to foodborne pathogens with purified extract could serve to formulate starter culture that can produce bioprotective *ergo*.

Keywords: *ergo*, lactic acid bacteria, foodborne pathogens, antagonism, bioprotective starter, safety.

INTRODUCTION

Fermented dairy products in Ethiopia include *ergo* (fermented milk), *ayib (*cottage cheese), *arerra* (sour butter milk) and k*ibe* (butter). *Ergo* or *ititu* is commonly produced by natural fermentation process of raw caw milk without addition of any defined starter cultures. It is simply a natural process which is carried out by the proliferation of the initial milk flora, with establishment and involvement of successive fermenting microbial groups as regulated by the chemical changes in the fermenting milk (Mogessie Ashenafi, 1996). Their metabolic action and dominance of lactic acid bacteria during fermentation of these products determine and maintains the taste, flavor, aroma, food quality and safety of the product.

Since *ergo* is mainly LAB fermented product; Kassaye *et al.* (1997) identified the predominant lactic acid bacteria as *Lactobacillus casei* and/or *Lactobacillus plantarum*. Mogessie Ashenafi (1995) indicated that during the fermentation of *ergo* the dominant lactic microflora were lactobacilli. Yoneya *et al.* (1999) reported *Lactococcus gravieae* and *Lactococcus lactis* subsp. *lactis* from samples of *ergo* as lactic acid producing lactococci.

Various workers established that the foundation of preservation and enhancement of the microbiological safety and quality of fermented products is mainly due to microbial antagonism (Leroy and De Vuyst, 1999; Ryan *et al*., 1996). The dominance of LAB during fermentation of fermented products and mainly their antagonistic activity against undesirable microorganisms including spoilage organisms and foodborne pathogen is primarily associated with resource competition; production of different low-molecular weight substances (like acetaldehyde, diacetyl, hydrogen peroxide, etc.); production of different organic acids; lowering pH, bacteriocin production; and releasing bacteriocin-like substances (Abdelabasset and Djamila, 2008; Whiteford *et al*., 2001; Mora *et al.*, 2000; Franz *et al*., 2000; Kunene *et al*., 2000; Callewaert and De Vuyst, 2000; Gänzle *et al*., 2000).

Currently, traditional fermented products are receiving new attention for their health promoting and disease preventing/curing effects, i.e., probiotic significance. Besides, traditional fermenting processes, especially those spontaneously and uncontrolled could be a valuable source of Lactic Acid Bacteria (LAB) (Hamama, 1992; El Soda *et al*., 2003). The microbiological characteristics of several fermented milk have been studied in Indonesia (Hosono *et al*., 1989), South Africa (Beukes *et al*., 2001) and Morocco (Hamama, 1992).

The consumption of various species of lactic acid bacteria (LAB) as live cells in the form of fermented products, freeze-dried cultures (pharmaceutical in the form of tablets, capsules or granules), or as enriched health food products (in the form of liquid or powder) has been associated with different health benefits in humans (de Roos and Katan, 2000). Generally, both *in vitro* and *in vivo* evaluation procedures are employed for primary screening and assessment of probiotic properties of LAB. Commonly, the *in vitro* probiotic qualities of LAB include the testing acid-and bile-tolerance, and antimicrobial activities against enteropathogens (Kosin and Rakshit,

2006). Animal models and GI tract simulation studies have also been employed for probiotic selection criteria as well (Charteris *et al.*, 1998). The main objectives of this study were to screen antagonistic LAB and assess their primary probiotic qualities that include acid-bile tolerance of antagonistic LAB isolates recovered from *ergo*.

MATERIALS AND METHODS

Bacterial strains and collections of samples of *ergo*

Bacterial cultures used as target test strains in this study were *Salmonella Typhimurium* DT104, *Escherichia coli* ATCC 25922, Shigella shigelea SH10-1 and *Staphylococcus aureus* ATCC 25923. All LAB isolates were from this study. Twenty *ergo* samples were collected from vending houses and/or kiosks around Kotebe, to the North East of Addis Ababa. The samples were kept under refrigeration condition until processed.

Isolation and morphological identification of lactic acid bacteria (LAB) from *ergo*

LAB were isolated by diluting 25 ml of samples of *ergo* in 225 ml of 0.1% sterile peptone-water. Further dilutions were made as desired using the same diluents. Appropriate dilution of *ergo* samples were spread plated on the surface of pre-dried duplicate plates of de-Mann, Rogosa and Sharpe (MRS) agar. The plates were incubated at 32^0C in anaerobic jar for 48 h and then lactic acid bacteria were counted and purified. Purified LAB colonies from MRS agar were classified to genus level using morphological, Gram staining, spore formation and catalase production.

Screening for antagonism

Spot test

Spot test were done by spreading 100 µl of test pathogen on the surface pre-dried duplicate modified MRS agar and then spotting 10 µl form 48 h growth (in modified MRS broth) of LAB on the same plates. All plates were incubated as indicated above.

Antagonistic substance extraction

Cell-free supernatants of LAB isolates with antagonistic properties against the test pathogens were prepared by centrifuging cultures at 5000 g for 30 minutes at 4^0C. The supernatants were adjusted to pH 7.0 with 5M NaOH, which were known as crude supernatants, and were filter-sterilized (0.22 µm, Millipore).

Activity spectrum analysis of extracts testing using well-diffusion assay

The test indicators grown overnight were inoculated into 4 ml of modified MRS soft agar (0.7% agar), mixed and then over-layed on the surface of modified MRS agar plates. After solidification, wells were made on over-layed modified MRS agar plates at ten sites with sterile 7mm diameter cork borer. The floors of wells were sealed with a drop of modified MRS soft agar (0.7% agar). Wells on duplicate plates were filled with 50 µl of each LAB strain cell free supernatant. A well filled with sterile distilled water will serve as control. The supernatant in wells were allowed to diffuse for 4 hours at 4^0C. Zone of inhibition was checked after incubating plates under anaerobic condition at 32^0C for 24 h following the protocol indicated in Enan *et al.* (1996).

Preparation of purified extracts using 40% ammonium sulfate precipitation

Crude extract found to be antagonistic against the test pathogens were purified by gradually saturating the extracts with 40% ammonium sulfate. Then the extracts were kept at 4^0C with periodical shaking for 30 minutes. The pellets after centrifugation were solubilized with 10 mM sodium phosphate buffer, pH 5.8. Then, antagonistic activity of the purified extracts was done using well-diffusion method as indicated above.

Acid-and-bile tolerance

The antagonistic LAB isolates were tested for acid-and-bile tolerance following the protocol given by Hyronimus *et al.* (2000). Log 6 cfu/ml of each overnight culture of LAB Isolates was inoculated into tubes of 10 ml MRS broth with pH values of 2.0, 2.5, and 3.0 adjusted using 3M HCl. Tubes incubated at 37^0C were checked on MRS agar for survival after 3 and 6 h. Plates were incubated at 37^0C for 24/48 h in anaerobic jar. Each candidate was further checked by streaking the organism onto MRS agar with respective pH values and incubating plates at 37^0C for 24 h. Survival percentage of each antagonistic LAB compared to the initial concentration was determined on MRS agar. LAB isolates with the survival rate ≥50% at pH 2.5 after 6 h were considered as acid-tolerant. The bile-tolerance of acid-tolerant antagonistic LAB isolates were examined by adding log 6 cfu/ml of each acid-tolerant LAB into 10ml of MRS broth containing O.3% oxgall Bile. All tubes were incubated at 37^0C and growth was checked at 24 and 48 h by spreading on MRS agar incubated at 37^0C in anaerobic jar.

RESULTS AND DISCUSSIONS

Screening antagonistic LAB against foodborne pathogens

A total of 500 LAB were recovered and purified on MRS agar from *ergo* samples collected from vendor shops around Kotebe, which is located in Yeka Sub-city, to the North East of Addis Ababa. The spot test screening showed that 41.8% (209) of LAB isolates were found inhibitory to the test pathogens (Table-1). Of the 209 LAB with inhibitory characteristics, 2.6%, 9.8%, 12% and 17.4% were observed inhibitory to all four, three, two and one test foodborne pathogens (*Salmonella typhimurium* DT104, *Escherichia coli* ATCC 25922, *Shigella shigelea* SH10-1 and *Staphylococcus aureus* ATCC 25923), respectively.

Table-1. Screening of lactic acid bacteria (LAB) for inhibition of foodborne pathogens.

Total no. of LAB tested	No. (%) found inhibitory*				Total no. (%) LAB found inhibitory to the test pathogens
	One test pathogen	Two test pathogen	Three test pathogen	Four test pathogen	
500	87(17.4 %)	60 (12 %)	49 (9.8 %)	13 (2.6 %)	209 (41.8%)

* The test pathogens were *Salmonella typhimurium* DT104, *Escherichia coli* ATCC 25922, *Shigella shigelea* SH10-1 and *Staphylococcus aureus* ATCC 25923

From 14 human bifidobacterium strains only 2 were shown with antagonistic activity during *in vitro* analysis against *Salmonella typhimurium* SL1334 by Lievin *et al.* (2000). Sobrino *et al.* (1991) also reported that from 720 LAB recovered from Spanish dry fermented sausage screened for antagonistic activity under conditions that eliminated the effect of low pH and H_2O_2 only 119 were found inhibitory to *Lab. fermentum* CECT285. Maciel *et al.* (2003) showed that from 484 LAB isolated from Italian *salami,* 45 isolates inhibited *Listeria monocytogenes*, *Staphylococcus aureus* (ATCC 25923), *Salmonella enteritidis* (ATCC 13076) and *Escherichia coli* (ATCC 25922) with larger inhibitory zone when tested by deferred method. From 52 LAB isolated from Algerian fermented milk 5 were shown to produce bacteriocin (Abdelbasset and Djamila, 2008).

Analysis of the antagonistic effect of extracts from LAB

Crude extracts of all 209 LAB isolates with antagonistic effect against one to all four test pathogens were tested with well-diffusion assay. From which 40 (19.1 %) LAB isolates were found inhibitory to the test pathogens with ≥ 10 mm inhibitory zones against foodborne pathogens with well diffusion assay (data not shown). Of these 40 LAB isolates, the crude extracts of 22 (55 %) of LAB isolates indicated to inhibit the test pathogens with ≥ 20 mm inhibitory zones with the same method (Table-2 and Figure-1). About 15%, 9.22%, 7.5% and 10% of this LAB were shown to be inhibitory to one, two, three and four test pathogens, respectively. Purified extracts from antagonist LAB were tested for inhibition of the same test foodborne pathogens. Of the crude extract, 72.7% of the purified extract was shown to be antagonistic to the test pathogens (Table-2). In a similar study**,** Pereze *et al.* (2001) reported that from 7 lactobacilli isolated from feces of cat and dog culture supernatant only 1 strain (*Lab. acidophilus* La1) was found inhibiting the proliferation of *Gardia intestinalis* trophozoites.

Table-2. The effect of crude extracts from antagonistic LAB against foodborne pathogens with well diffusion assay.

Total no. of LAB tested	No. (%) LAB found inhibitory to the test pathogens	No. (%) found inhibitory			
		One	Two	Three	Four
Crude extract from 40 LB isolates	22 (55 %)	6 (15 %)	9 (9.22 %)	3 (7.5 %)	4 (10 %)
ASE* from 22 LAB isolates	16 (72.73 %)	1 (15 %)	4 (9.22 %)	9 (7.5 %)	2 (10 %)

* ASM = Ammonium sulfate extract

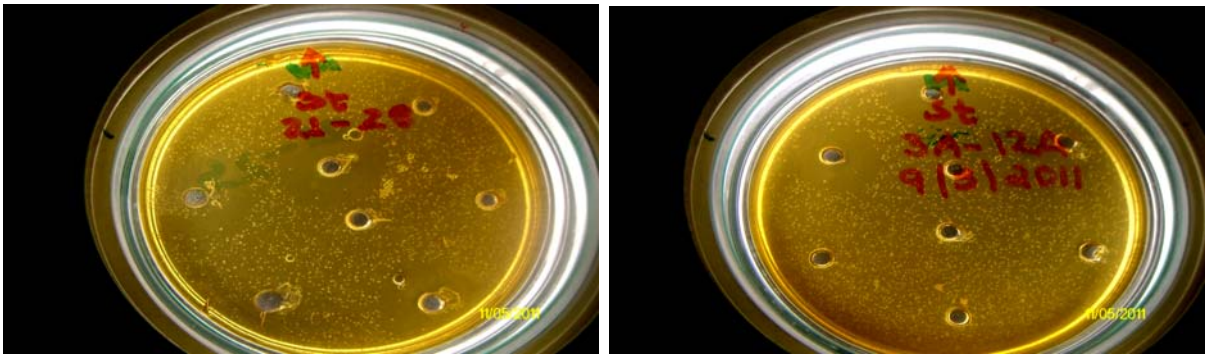

Figure-1. Inhibitory zone formed by the purified extracts from the antagonist LAB against one the foodborne pathogens *Salmonella typhimurium* DT104.

Acid-and-bile tolerance

Only 7 LAB isolates from 16 antagonistic LAB survived exposure to pH 2.5 for 3 hours and further incubation to 6 hours (Table-3). None of 16 LAB isolates with antagonistic characteristics against the test pathogens were survived exposure to pH 2.0 for 3 hours. About 14 isolates tolerated pH 3.0 for 3 h. Further incubation for 6 h reduced the survivors to 10. The 7 LAB isolates those survived exposure to pH 2.5 were also shown tolerant to 0.3% bile for 48 h. These 7 LAB isolates (the acid-bile tolerant), can possibly survive in stomach of the human host, the 'first hurdle' and 'second hurdle' the intestine. Likewise, Jacobsen *et al.* (1999) selected 5 isolates from 47 selected strains based on *in vitro* qualities. Corcoran *et al.* (2005) also indicated that different *Lactobacillus spp.* exhibited different rate of survival at pH 2.0 for 90 minutes, and considered those with relatively higher count as good candidates.

Table-3. Acid-bile tolerance of antagonistic LAB.

LAB isolates	Count in log cfu/ml (survival rate [%]) of LAB						
	pH 2.0	pH 2.5		pH 3.0		Bile	
	After 3 Hrs	After 3 Hrs	After 6 Hrs	After 3 Hrs	After 6 Hrs	After 24 Hrs	After 48 Hrs
Lactobacillus 1	0 (0)	<1 (51)	3.82 (61)	2.57 (39)	1.84(28)	+++	++
Lactobacillus 2	0 (0)	1.60 (23)	<1 (16)	3.59(55)	2.76 (45)	++	++
Lactobacillus 3	0 (0)	<1 (67)	0 (0)	4.05 (62)	3.66 (56)	++	++
Lactobacillus 4	0 (0)	5.19 (74)	3.77 (54)	3.85 (59)	2.74 (42)	+++	+++
Lactobacillus 5	0 (0)	<1 (3)	<1 (5)	3.92 (60)	3.01 (46)	++	++
Lactobacillus 6	0 (0)	4.60 (66)	3.58 (51)	5.03 (77)	4.11 (63)	+++	+++
Lactobacillus 7	0 (0)	<1 (5)	<1 (1)	3.27 (50)	2.61 (40)	++	++
Lactobacillus 8	<1 (2)	4.19 (81)	3.45 (57)	5.94 (91)	4.38 (67)	+++	++
Lactobacillus 9	0 (0)	3.76 (54)	3.42 (49)	5.75 (88)	4.33 (67)	+++	+++
Lactobacillus 10	0 (0)	4.26 (55)	3.56 (51)	3.92 (60)	3.72 (57)	+++	+++
Lactobacillus 11	0 (0)	<1 (2)	0 (0)	2.68 (41)	2.09 (32)	+++	+++
Lactobacillus 13	0 (0)	<1 (5)	0 (0)	4.23 (65)	3.59 (55)	++	++
Lactococcus 1	1.36 (12)	4.20 (69)	3.28 (54)	5.42 (83)	3.66 (56)	+++	++
Lactococcus 2	<1 (5)	<1 (3)	0 (0)	5.16 (78)	3.86 (59)	++	++
Lactococcus 3	<1 (12)	4.88 (76)	4.04 (62)	5.16 (79)	4.51 (69)	+++	++
Lactococcus 4	<1 (1)	<1 (20)	0 (0)	3.46 (53)	3.20 (49)	+++	++

* = Selected for in vivo probiotic tests, +++ = survived with a count of >log7 cfu/ml, ++= survived with a count between log6 and 7 cfu/ml

Application of antagonistic LAB starter is important to prevent not only the outgrowth of food pathogens and spoilage organisms during fermentation process; it is also shown useful during post-fermentation storage of the products. The rural and urban people in Ethiopia are still commonly use unpasteurized fermented milk for production of *ergo* and other fermented milk products. Possible use of probiotic lactic starter cultures for production of safe and biopreservative *ergo* was suggested by Tesfaye *et al*. (2011). The result of this study displayed that our 7 pure LAB isolates demonstrated remarkable *in vitro* probiotic potential. The *in vitro* results strongly suggest that the isolates are promising good candidate probiotics that can be used for the formulation of starter cultures for preparation of safe fermented products.

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