ORIGINAL ARTICLE

IN-VITRO PROBIOTIC POTENTIAL OF LACTIC ACID BACTERIA ISOLATED FROM 'WAKALIM', A TRADITIONAL ETHIOPIAN FERMENTED BEEF SAUSAGE

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ABSTRACT

BACKGROUND: ***Probiotics are live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance. To be used as a probiotic, a bacterial strain is required to have good tolerance to acidity of the stomach and bile salt of the upper small intestine. Report on the probiotic efficacy of lactic acid bacteria isolated from traditional Ethiopian fermented foods, including 'wakalim', is scanty. Although lactic acid bacteria are known for their probiotic potential, the probiotic efficiency of Lactobacillus species are strain specific. The objective of this study was to evaluate the probiotic potential of strains of lactic acid bacteria isolated from 'wakalim', a traditional Ethiopian fermented beef sausage.***

MATERIALS AND METHODS: ***In this study, the in-vitro probiotic potentials of lactic acid bacteria isolated from fermenting 'wakalim' were evaluated. 'Wakalim' was prepared following traditional techniques. Strains of lactic acid bacteria isolated from 'wakalim' and evaluated for their probiotic potential were identified to species level using API 50CHL kits. The strains were investigated for tolerance to acidity (pH=2.0, 2.5, 3.0) and bile salt (oxgall) concentrations ranging from 0.3%-1.0% following the standard procedures.***

RESULTS: ***Of the total 99 strains of lactic acid bacteria isolates, 44 (44.0%) tolerated pH 3.0 and bile salt concentration ≥ 0.3% for 3 hours. The highest tolerance to pH 3.0 was observed among the pediococci (81.6%, 31/38) followed by lactobacilli (14.3%, 8/56). All the few isolates of lactococci, Leuconostoc, and Weissella were also tolerant to pH 3.0. The difference in resistance to low acidity and bile salt concentration (≥ 0.3%) of pediococci was statistically significant ($p<0.05$) from the Lactobacillus species***.

CONCLUSIONS: ***Strains of lactic acid bacteria isolated from traditional Ethiopian fermented sausage have promising in-vitro probiotic potential. Therefore, consumption of fermented sausages could have health enhancing effect.***

KEY WORDS: ***Fermented sausage, Lactic Acid Bacteria, Probiotics, Ethiopia***

INTRODUCTION

Probiotic bacteria have been defined as "live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance" (1). The use of live microorganisms for enhancement of consumers' health, as an aid to cure some types of gastro-intestinal disorders has been recognized for almost a century since the first report by Mitchenkov in 1910 (2). However, the utilization of health aspects of food products and their marketing began in the 1960s. Currently, specific functional food products have scientifically proven to benefit the health and well-being of consumers. Proposed functional foods in Europe include 60% of dairy products, 25% fat-based spreads, 10% bakery and cereal products, and 5% drinks (3).

Microbes belonging to different genera have been used as probiotics. Among these, the probiotic properties of lactobacilli and bifidobacteria are the best studied and, thus, are the most common in probiotic products designed for human use (4). Having long history of safe use in foods, most lactic acid bacteria (LAB), including members of the genera *Lactococcus* and *Lactobacillus* are given the generally regarded as safe (GRAS) status.

Several health benefits have been proposed for probiotic bacteria. The health benefits supported by adequate clinical data or promising animal data include prevention and treatment of diarrheal disease, prevention of systemic infections, management of inflammatory bowel disease, immunomodulation, prevention and treatment of allergies, anticancer effects, treatment of cholestrolaemia, and alleviation of lactose intolerance (5, 6, 7).

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Lactic acid bacteria that dominate the lactic flora in the final product of 'wakalim' fermentation are consumed along the product and could have some health enhancing potential. Report on the probiotic efficacy of LAB isolated from traditional Ethiopian fermented foods, including 'wakalim', is scanty. The aim of this study was, therefore, to evaluate the probiotic potential of some LAB isolated from 'wakalim', a traditional Ethiopian fermented beef sausage.

MATERIALS AND METHODS

'Wakalim' is a traditional Ethiopian fermented beef sausage commonly produced and consumed in the eastern part of the country mainly among the Harari and Argoba tribes. This study was conducted at Addis Ababa University, Aklilu Lemma Institute of Pathobiology in 2006.

Strains used in this study were isolated from fermenting 'Wakalim' prepared following the traditional fermentation procedure as described earlier (Fig.1) (8). Sample (25 g 'wakalim') was drawn every 24 hours for seven days to the end of fermentation and homogenized in 225 ml of sterile peptone-water (0.1%) for 2 minutes using a Stomacher lab blender (Stomacher 400, Seward, London, UK). 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, GasPak Anaerobic Systems) at 30 to 32°C for 48 hours. 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 identification. Colonies of LAB were transferred into about 5ml MRS broth (Oxoid) and purified by repeated streaking on MRS agar. The pure cultures were streaked on slants of MRS agar and were stored at 4^0C for further biochemical characterization.

The purity of isolates was confirmed through microscopic observation for homogeneity. Gram-positive, catalase-negative, non-spore forming isolates were considered as LAB and further characterized for their carbohydrate fermentation profile using API 50CH kit (Biomeriuex, Marcy I'Etoile, France). The biochemical profiles of isolates were analyzed to species and subspecies level using identification software (API WEB, V 1.1.0, Biomeriuex, Marcy I'Etoile, France).

A total of 99 strains of LAB, comprising species of the genus's *Pediococcus* (38), *Lactobacillus* (56), *Lactococcus* (3), *Leuconostoc* (1), and *Weissella* (1) were used.

Acid tolerance of the isolates was determined as per procedures described earlier (9). Briefly, cultures were grown in MRS broth at 37 ^{0}C overnight and 1ml was separately sub-cultured in 10ml of fresh MRS broth adjusted to pH values of 2.0, 2.5, or 3.0 using hydrochloric acid (3.0 M) to simulate the gastric environment. The initial bacterial concentration after inoculation was about 10^6 cfu/ml and this was checked by viable count determination on MRS agar. Samples were incubated for 3 and 6 hours at 37^0C. After appropriate incubation, 1ml of the culture was diluted in pre-sterilized 9 ml phosphate buffer (Sigma, St. Louis, USA) prepared according to the manufacturer's instruction (0.1 M, pH 6.2) in order to neutralize the medium acidity. The residual viable count was determined after further serial dilution in 0.1% buffered peptone water (BPW) and plating 0.1ml aliquot of appropriate dilution on MRS agar. Culture-free MRS 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 hours 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.

Those isolates that survived the acid treatment were further characterized for their minimal inhibitory concentration (MIC) with respect to bile salt as previously described (9). Samples of overnight cultures (20 µl corresponding to $2x10^6$ cfu/ml) were spotted onto pre-dried surfaces of MRS agar plates supplemented with different concentration levels of bile salt (0.1 to 1% w/v oxgall, a total of 10 dilutions) (Sigma Chemical Co. St Louis, Missouri, USA). Plates were incubated for 3 to 5 days. The 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 MRS broth plated on MRS agar plate was used as a control. Those isolates that tolerated concentrations of bile salt over 0.3% were considered for further analysis.

The bile tolerance of isolates was determined according to the methods described earlier (10). Two portions of MRS broth were prepared: one portion was supplemented with 0.3% (w/v) bile salt and the other portion free of bile salt. The bile supplemented MRS broths were separately inoculated with an overnight culture of the test LAB strains to a level of 10^6 cfu/ml. The Non-bile supplemented MRS broth inoculated with same amount of test strains were used as control. All samples were incubated at 37 ^{0}C for 8 hours. Growth was monitored at an interval of 1 hour by measuring absorbance at 600 nm using spectrophotometer (Jenway ltd, 6405 uv/vis, UK). Coefficient of inhibition (C_{inh}) was calculated using the method described by Gopal et al (11):

C_{inh} = (ΔT_8-T_0 Control - ΔT_8-T_0 Treatment)/ (Δ T_8-T_0 Control)

Where, Δ represented the differences in absorbance between T_0 (zero hours reading) and T_8 (reading on the 8th hour). The experiment was done twice to ensure the reproducibility of the result.

Based on calculated coefficient of inhibition (C_{inh}), isolates were classified into non-sensitive (resistant) to 0.3% bile salt ($C_{inh} \approx 0$), with retarded growth ($0.2 < C_{inh} < 0.4$), and poorly tolerant ($C_{inh} > 0.4$).

Data were entered in to computer and analyzed using SPSS for Windows version 10.0. The significance of differences ($P<0.05$) among isolates with respect to degree of tolerance to acidity and bile salt was compared using one-way ANOVA.

RESULTS

Two genera of LAB, namely *Lactobacillus* and *Pediococcus* dominated the lactic flora towards the end of wakalim fermentation (Table 1).

Findings on acid tolerance of isolates showed that 81.6% (31/38) of pediococci, and 14.3% (6/56) lactobacilli could tolerate pH 3.0 for three hours. Further extension of the incubation period for 3 more hours, however, slightly reduced the number of survivors of pediococci. As a result, 71.1% (27/38) of the pediococci were tolerant to acidity with 90 to 100% survival rate. At any rate, the degree of tolerance of pedococci to acidity was significantly different ($P<0.05$) from that of lactobacilli. The few representative species of the genus *Lactococcus, Leuconostoc* and *Weissella* survived for 6 hours at pH 3.0 (Table 2).

Ninety two (92.9 %) of the total tested isolates did not survive exposure to pH 2.5 for 3 hour. The survival rates of 79 Isolates (79.8%) were below 10% after 3 hours of incubation in the same pH environment. Only 5 (13.2%) isolates of the pediococci, mainly *Ped. pentosaceus* 1, and two isolates of lactococci, both belonging to *Lactococcus lactis* spp. *lactis*, managed to survive at pH 2.5 for 3 h with 100% survival rate (Table 2). None of the lactobacilli was recovered from pH 2.5 after 3 hours incubation. At pH 2.0, almost all strains did not survive the stringent acidity of the simulated gastric medium.

Eighty three percent (44/53) of the isolates, formerly found tolerant to pH 3.0, survived bile salt concentration above 0.5%. While 34.0% (18/53) of the isolates had MIC of bile salt above 1%, 17.0% (9/53) had below 0.3% tolerance to bile salt. Pediococci were the most tolerant to bile salt concentration above 1% followed by lactobacilli, with proportion of 37.9% (11/29) and 15.8% (3/19), respectively. Among the few isolates of lactococci, Leuconostoc, and Weissella sp., strains of lactococci had better tolerance to bile salt (Table 3). Of isolates of lactobacilli, strains of Lb. fermentum were the most sensitive to bile salt with concentration above 0.5%, while *Ped. pentosaceus* 1 were the most tolerant species of the genus Pediococcus.

A total of 33 isolates with MIC of bile salt greater than 0.3% were further evaluated for their tolerance to the same bile salt concentration (MRS broth supplemented with 0.3% bile salt) under extended incubation for 8 hours. Some discrepancies were observed between results of MIC study and measurement of absorbance (A_{600}). In MIC study, all the 24 isolates of *Ped. pentosaceus*1 (100%), and few (3 isolates) of the *Ped. Pentosaceus* 2 tolerated bile salt greater than 0.3%. However, when 13 of the *Ped. Pentosaceus* 1 isolates with relatively higher MIC ($\geq$ 0.7%) were further examined under extended incubation, 9 of them were found poorly tolerant to 0.3% bile salt as determined by measurement of absorbance (A_{600}) for 8 hours (Cinh > 0.40). One strain each of *Lb. pentosus* and *Ped. pentosaceus* 2 and three strains of *Ped. pentosaceus* 1 had good tolerance to the tested bile salt concentration with some degree of retardation in their growth rate ($0.2 < Cinh < 0.4$) (Table 4).

In general, out of the 99 strains of LAB originally evaluated for their in-vitro probiotic potential, 44.4% (44/99) tolerated acidity of pH 3.0 for 3 hours and also tolerated bile salt above 0.5%. Moreover, about 8 (8%) isolates remained viable under extended incubation period of 8 hours in 0.3% bile salt concentration. Those few isolates that resisted the extended incubation were non-sensitive (resistant) to 0.3% bile salt ($Cinh \approx 0$) included one strain each of W. viridescens, Lb. plantarum1, Lb. pentosaceus, Ped. pentosaceus2, and four isolates of Ped. pentosaceus1 (Table 4).

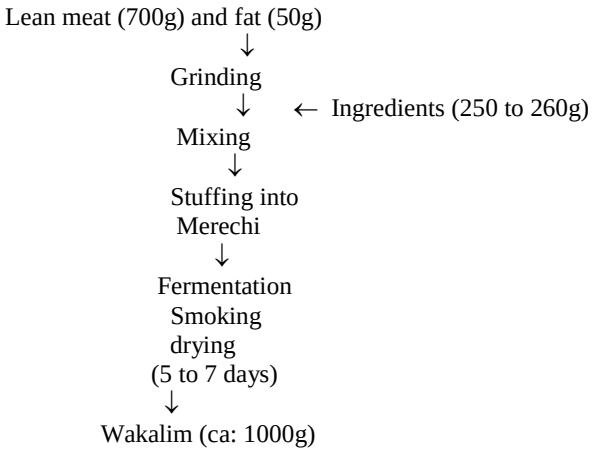

Figure 1. Flow diagram for the production of 'wakalim'

Table 1. Frequency distribution (log cfu/g) of LAB that dominate the final product of 'wakalim' fermentation, Addis Ababa, 2006

Fermentation period (hour)	Count (log cfu/g) of LAB	Relative proportion (log cfu/g) of different species of genera of LAB							
		Pediococcus pentosaceus	*Lactobacillus plantarum*	*Lb. pentosus*	*Lb. delbrueckii spp.delbrueckii*	*Lb. brevis 3*	*Lb. fermentum*	*Unidentified Lactobacillus*	*Unidentified Pediococcus*
0	5.96	0	0.09	0.00	0.09	0.21	0.30	3.87	0.89
12	9.35	0	0.00	0.00	0.00	0.00	0.00	6.55	2.81
24	9.53	1.29	0.48	0.14	0.14	0.00	0.14	3.81	3.19
36	9.57	3.35	0.00	0.00	0.00	0.00	0.00	4.30	0.00
48	9.6	1.78	0.14	0.48	0.00	0.00	0.45	1.92	1.49
72	9.62	1.78	0.00	0.14	0.00	0.48	0.34	1.15	1.44
96	9.65	1.44	1.45	0.14	0.48	0.48	0.82	1.78	1.78
120	9.55	2.90	2.39	0.14	0.48	0.33	0.33	4.15	0.33
144	9.65	0.53	2.90	0.34	0.34	0.15	0.00	3.37	2.89

Where, LAB= Lactic acid bacteria; cfu= colony forming unit; Lb= Lactobacillus

Table 2. Survival rate (%) patterns of isolates of LAB under different acidic condition after 3 and 6 hours exposure, Addis Ababa, 2006

Isolates	No. of isolates	pH = 3.0									pH = 2.5						pH = 2.0	
		3.0 h					6.0 h				3.0 h			6.0 h			3.0 h	
		% Survival																
		< 1	1-10	30-40	60-75	100	<1	1-20	35-65	90-100	<1	1-10	100	<1	1-10	100	<1	1-10
Pediococci	38	0	2	3	2	31	1	4	3	27	10	9	5	5	2	2	3	12
Lactobacilli	56	27	15	5	2	8	0	11	2	8	27	10	0	3	0	0	1	3
Lactococci	3	0	0	0	0	3	0	0	0	3	0	0	2	1	0	0	0	0
Leuconostoc	1	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0
Weissella	1	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	1
Total isolates	99	27 (27%)	17 (17%)	8 (8%)	4 (4%)	44 (44%)	1 (1%)	15 (15%)	5 (5%)	40 (41%)	38 (38%)	20 (20%)	7 (7%)	9 (9%)	2 (2%)	2 (2%)	4 (4%)	16 (16%)

DISCUSSION

Evaluation of probiotic potential of LAB isolates was carried out by simulating the environment in the gastro-intestinal tract (GIT): subjecting the isolates to pH 2.0 to 3.0, exposure to bile salt 0.3% (w/v) concentration, and incubation for at least 3 hours. The typical transit time of food in the stomach is approximately 20 minutes to 3 hours (9,12). Among other factors, stomach acidity varies from person to person naturally and whether an individual has fasted prior to ingestion or not (12). We thus investigated the survival ability of our test strains at various acidic pH values.

Higher number of *Pediococcus* species and some strains of lactobacilli showed promising probiotic potential. The resistance to low acidity and bile salt concentration (≥ 0.3%) of pediococci was statistically significantly different from what was observed among the *Lactobacillus* species. Being member of the homo-fermentative strains of LAB, pediococci have already adapted to acid environment. Except one facultatively and another obligately heterofermentative species, all pediococci were homo-fermentative, producing lactate from glucose and no gas (7).

Table 3. Determination of minimal inhibitory concentration (MIC*) of isolates with respect to bile salt, Addis Ababa, 2006

Isolates	No. of isolates	Number of isolates whose MIC* of bile salt (%) is as follows:								
		≤ 0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	>1
Pediococci	29	1	0	1	0	3	4	4	5	11
Lactobacilli	19	8	0	1	0	1	3	1	1	4
Lactococci	3	0	0	0	0	1	0	0	0	2
Leuconostoc	1	0	0	0	0	0	0	0	1	0
Weissella	1	0	0	0	0	0	0	0	0	1
Total isolates	53	9 (17%)	0	2 4%)	0	5(9%)	7(13%)	5(9%)	7(13%)	18(34%)

* MIC is defined as the lowest concentration of bile salt that totally inhibited the growth of bacteria.

High acidity in the stomach and the high concentration of bile components in the proximal intestine are the major host factors that a probiotic strain should tolerate to express probiotic effect on the host. More than 44 % of the isolates had 100% survival for 3 hours in MRS broth with pH=3.0. However, only seven isolates had 100% survival at pH 2.5. Similar study reported complete loss of viability in strains of lactobacilli, including *Lb. casei* and *Lactobacillus* GG, at pH 2.5 (10). Other study as well reported that none of their 44 *Lactobacillus* strains tested for tolerance were capable of replication at pH 2.5 (13). On the other hand Mishera *et al* reported 3 of their 7 isolates of *Lb. casei* tolerated pH 2.0 and/or 3.0 (14). When compared to the weak tolerance to low pH seen in some LAB investigated in earlier studies, our isolates had higher survival rate at pH 3.0 and pH 2.5. Although acid tolerance in lactobacilli is highly strain specific, members of the genus *Lactobacillus* are, in general, aciduric or acidophilic, producing pH 4.0 in foods containing a fermentable carbohydrate (15). The probiotic effect of LAB may partly be based on the production of relevant concentrations of lactic acid in the microenvironment, which, in combination with detergents like bile salts, inhibits the growth of Gram-negative pathogenic bacteria (7).

Although what was observed in-vitro may not necessarily reflect the case in-vivo, high rate of survival of our isolates was an indication of their possible survival in stomach of the human host before their transit to the small intestine. In addition, their resistance to 0.3% bile salt concentration is indicative of their possible survival in the small intestine as observed in LAB isolated from Greek dry fermented sausages (16).

Resistance to bile salt of our isolates could be attributed to their ability to produce bile hydrolase (17). Bile salt hydrolase (BSH) protects the cells that produce it from the toxicity of conjugated bile salts by deconjugating the bile acids (18). Compared with their conjugated counterparts, deconjugated bile acids have decreased solubility and diminished detergent activity and may, therefore, be less toxic to bacteria in the intestine (19).

The survival rate of our isolates in stomach and small intestine could be much better than what was observed in-vitro. Intake of these potentially probiotic LAB along with fermented 'wakalim' could give these isolates some protection during their transit along the GIT. The presence of proteins, lactose or other oligosaccharides in commercial probiotic products is known to improve the survival of probiotic microorganisms in the small intestine (10, 19). Thus, 'wakalim', rich in beef protein could give better protection to the potentially probiotic isolates on their way through the gastric environment and establishment in the small intestine. The detrimental effect of gastric fluid on probiotic bacteria can be hampered when the probiotic organisms are consumed with food (20) as , consumption of probiotics along food like sausage could raise the pH and reduce the adverse effect of gastric acidity.

As observed from the total counts of LAB in the course of 'wakalim' fermentation, the numbers of viable strains in the final product (10^8 to 10^9 cfu/g) were within the estimated minimum dose of daily ingestion of probiotic

bacteria (10^9 to 10^{10} viable microbes in dry fermented sausage) although the minimal dose is dependent on several factors, such as individual variability, strain and food product (3).

In conclusion, LAB of 'wakalim' origin have shown promising in-vitro probiotic potential that needs further in-vivo characterization using animal models.

Table 4. Bile salt (0.3%) tolerance pattern of some strains of LAB isolated from 'wakalim', Addis Ababa, 2006

Isolates code	Isolates likely strain	Mean Coefficient of inhibition (Cinh)	Isolates category (A, B, C)* with respect to bile tolerance
MR408	Lactobacillus plantarum1	0.03	A
MR 422	Weissella viridescens	0.08	A
MR 262	Pediococcus pentosaceus 1	0.14	A
MR 266	Ped. pentosaceus 1	0.22	B
MR 239	Ped. pentosaceus 1	0.26	B
MR 280	Ped. pentosaceus 1	0.29	B
MR 249	Lb. pentosaceus	0.40	B
MR 435	Ped. pentosaceus 2	0.40	B
MR 489	Lb. plantarum 1	0.41	C
MR 457	Lb. brevis1	0.41	C
MR 440	Ped. pentosaceus 1	0.42	C
MR 405	Lb. plantarum1	0.43	C
MR 279	Ped. pentosaceus 1	0.45	C
MR 513	Ped. pentosaceus 2	0.46	C
MR 500	Ped. pentosaceus 2	0.48	C
MR 1201	Pediococcus species	0.50	C
MR 425	Lactococcus lactis spp. lactis	0.50	C
MR 423	Lact. Lactis spp. lactis	0.50	C
MR 427	Lact. Lactis spp. lactis	0.56	C
MR 511	Ped. pentosaceus1	0.60	C
MR 508	Ped. pentosaceus 1	0.61	C
MR 247	Ped. pentosaceus 1	0.62	C
MR 283	Ped. pentosaceus 1	0.64	C
MR 268	Ped. pentosaceus 1	0.65	C
MR 1446	Lb. fermentum	0.70	C
MR 272	Ped. pentosaceus 1	0.70	C
MR 483	Lb. pentosus	0.70	C
MR 242	Ped. pentosaceus 1	0.80	C
MR 727	Lactobacillus species	0.82	C
MR 966	Lactobacillus species	0.86	C
MR 1442	Lb. plantarum 1	0.87	C
MR 390	Lb. paracasei spp. paracasei	0.93	C
MR 430	Leuconostoc lactis	0.96	C

* A = not-sensitive (resistant) to 0.3% bile salt (Cinh ≈ 0), B = with retarded growth (0.2< Cinh < 0.4), and C = poor tolerance (Cinh > 0.40); Isolates of category 'A' and 'B' show strains with relatively better in-vitro probiotic potential.

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REFERENCES

1. Fuller R. Probiotics in man and animals. A review. *J Appl Bacteriol* 1989; 66:365-378.
2. Hawley HB, Sheperd PA, Wheather DM. Factors affecting the implantation of lactobacilli in the intestine. *J Appl Bacteriol*, 1959; 22:360-367.
3. Tyopponen, S Petaja E, Mattila-Sandholm T. Bioprotectives and probiotics for dry sausages: A review. *Int J Food Microbiol*, 2003; 83:233-244.
4. Tannock GW. Analysis of the intestinal microflora: a renaissance. *Antonie Van Leeuwenhoek*, 1999; 76:265-378.
5. Spahaak S, Havenaar R, Schaafsma G. The effect of consumption of milk fermented by *Lb. casei* strain shirota on the intestinal microflora and immune parameters in humans. *Eur. J Clin Nutr*, 1998; 52:899-907.
6. Gill HS, Guarner F. Probiotics and human health: a clinical perspective. *Postgrad Med J*, 2004; 80:16-26.
7. Stiles ME, Holzapfel WH. Lactic acid bacteria of foods and their current taxonomy. *Int J Food Microbiol*, 1997; 36:1-29.
8. Ketema Bacha. Microbiological study of wakalim, a traditional Ethiopian fermented sausage. PhD Dissertation, Department of

Biology, Addis Ababa University, Ethiopia. 2007:180pp.

9. Hyronimus B LeMarrec C Haji Sassi A Deschamps A. Acid and bile tolerance of spore-forming lactic acid bacteria. *Int J Food Microbiol*, 2000; 61:193-797.
10. Charteris WP, Kelly PM, Morelli L, Collins JK. Development and application of in-vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *J Appl Microbiol*, 1998; 84:759-768.
11. Gopal PK, Prasad J, Smart J, Gill HS. In-vitro adherence properties of *Lb. rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *E. coli. Int J Food Microbiol*, 2001; 67:207-216.
12. Goldin B, Gorbach S, Saxelin M, Barakat S, Gualtieri L, Salminen S. Survival of *Lactobacillus* species (Strain GG) in human gastrointestinal tract. *Digestive Dis Sci*, 1992; 37:121-128.
13. Jacobsen CN, Rosenfeldt NV, Hayford AE, Moller PL Michaeken KF, Paerragaard A, Sandstrom B, Tvede M, Jakobsen M. Screening of probiotic activities of forty seven strains of Lactobacillus spp. by in-vitro techniques and evaluation of colonization ability of five selected strains in human. *Appl Environ Microbiol*, 1999; 65:4949-4956.
14. Mishera V, Prasad DN. Application of in-vitro methods for selection of *Lactobacillus casei* strains as potential probiotics. *Int J Food Microbiol*, 2005; 103:109-115.
15. Alakomi HL, Skytta E, Saarela M, Mattila-Sandholm T, Latva-Kala K, Helander I. Lactic acid permeabilizes Gram-negative bacteria by disturbing the outer membrane. *Appl Environ Microbiol*, 2000;66:2001-2005.
16. Papamanoli E, Tzanetakis N, Lipopoulou-Tzanetaki E, Kotzekidou P. Characterization of lactic acid bacteria isolated from a Greek dry fermented sausage in respect of their technological and probiotic properties. *Meat Sci*, 2003; 65:859–867.
17. Savage DC. Gastrointestinal microbial ecology: possible modes of action of direct-fed microbials in animal production - a review of the literature. In: Direct-Fed Microbials in Animal Production. National Feed Ingredients Association, Iowa, 1992: pp. 11-81.
18. Walker DR, Gilliland SE. Relationship among bile tolerance, bile salt de-conjugation, and assimilation of cholesterol by *Lactobacillus acidophilus*. *J Dairy Sci*, 1993; 76: 956- 961.
19. Fernandez MF, Boris S, Barbes C. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. *J Appl Microbiol*, 2003; 94:449-455.
20. Incze K. Dry fermented sausages. *Meat Sci*, 1998; 49:S169–S177.