

ORIGINAL ARTICLE

ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF LACTIC ACID BACTERIA ISOLATED FROM ERGO, A TRADITIONAL ETHIOPIAN FERMENTED MILK, JIMMA, SOUTH WEST ETHIOPIA.**Abdulkadir Beyan* Tsige Ketema* & Dr. Ketema Bacha****Abstract*

Currently, the efficacies of antimicrobials have been threatened due to the development of resistance to antibiotics by some microorganisms. Lactic acid bacteria (LAB) from fermented products, may act as reservoir of antimicrobial resistance-genes that could be transferred to pathogens, either in the food matrix or in the gastrointestinal tract. The objective of this study was to evaluate the current resistance or susceptibility patterns of lactic acid bacteria isolated from Ergo served in Jimma Town, Southwest Ethiopia. A total of 57 isolates of LAB were identified to genus level following standard microbiological techniques. In this study, all 57 strains of lactic acid bacteria isolated from fermented Ergo samples were evaluated for their antimicrobial resistance patterns. A total of six pre-determined antibiotic discs (Oxoid) were used and the sensitivity tests were done following the modified standard Kirby –Bauer procedure. All the 57 tested lactic acid bacteria isolates were sensitive to Pencillin G (Pen, 10units), and Erythromycin (Ery, 15 µg). The most frequent resistance was noticed for methicillin (Met) (89.5%), followed by resistance to Norfloxacin (Nx) (80%). More than half of the isolates (54.38%) were resistant to two antimicrobials (Nx/Met), followed by resistance to only Methicillin (17.5%) and only Norfloxacin (15.8%). Although with low frequencies, a total of two multiple drug resistance (MDR) patterns (Ak/Nx/Met, and Tet/Ak/Nx/Met) were observed. In general, the isolates of LAB were not found reservoirs for transferable resistance genes for Penicillin G and Erythromycin as indicated by the sensitivity of isolates. Therefore, LAB isolated from traditional Ethiopian fermented food could still be used for the enhancement of consumer's health with periodic monitoring of the existing level of drug resistance.

Key words: Drug resistance, Ergo, Ethiopia, Jimma, LAB

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INTRODUCTION

The lactic acid bacteria (LAB) comprise a clade of gram positive, acid tolerant, generally non-sporulating, non respiring rod or cocci grouped together based on their common metabolic and physiological characteristics (Stiles and Holzapfel, 1997). They are usually found in decomposing plants and lactic products, producing lactic acid as major metabolic end product of carbohydrate fermentation. Such traits have linked lactic acid bacteria to food fermentation, as acidification of food products inhibit the growth of spoilage agents (Jay, 2000).

Some species of LAB commonly used in the food industry or naturally occurring in raw food materials are resistant to glycopeptides antibiotics including Vancomycin (Tynkkynen et al, 1998). Genes conferring resistance to several antimicrobials (including Chloroamphenicol, Erythromycin, Streptomycin, Tetracycline, and Vancomycin) located on transferable genetic elements (plasmids and transposons) have already been characterized in lactococci (Perreten et al., 1997) and lactobacilli (Gfeller et al., 2003) from foods. Resistance to common antibiotics by LAB could be intrinsic or acquired (Mathur and Sigh, 2005). Lactobacilli, pediococci and leuconostoc spp. have been reported to have a high natural resistance to vancomycin. The resistance could be due to the presence of

D-ala-D-lac as the normal peptide in their peptidoglycan (Florez et al., 2005). For a number of lactobacilli, a very high frequency of spontaneous mutation to kanamycin and streptomycin was reported (Curragh and Collin, 1992).

Currently there are lots of data on the prevalence of antibiotic resistance and the mechanisms implicated in clinical bacteria, but information about the antibiotic susceptibility/or resistance in LAB isolated from food is limited (Catalouck and Gogebaken, 2004; Florez et al., 2005).

Very recently, Ketema Bacha et al (2010) reported the susceptibility patterns of selected LAB isolated from wakalim, a traditional Ethiopian fermented beef sausage. Those strains isolated from traditional fermented foods could have application as a starter cultures for large scale production of the traditional product and/or as probiotic to enhance health of the consumers. But strains intended for use in the food systems as a starters of probiotic should be carefully examined for antimicrobial susceptibility (Teuber et al., 1999). The aim of this study was therefore, to evaluate the current resistance or susceptibility patterns of LAB isolated from ergo. Ergo is traditional Ethiopian fermented milk whose fermentation is largely dominated by both aerobic mesophilic bacteria (AMB) and lactic acid bacteria (LAB).

MATERIALS AND METHODS

Sample collection and processing. A total of 50 ergo samples were collected from cafés vending milk products in Jimma Town from February to June, 2009. Samples were collected on availability basis as the number of food establishments vending ergo on regular basis were not in much excess of the number of samples included in this study based on preliminary survey made before the resumption of the actual data collection. Accordingly, Samples of ergo (200ml each) were collected using sterile flasks. About 25 ml of the samples were aseptically homogenized separately in 225ml of sterile saline solution (0.85%) for 2 minutes using vortex mixer. Appropriate serial dilutions were made by transferring 1ml of homogenate into 9ml of sterile saline solution.

Plating and Enumeration: About 0.1 ml of appropriate dilution of the homogenized sample was surface plated in duplicates on pre-dried surface of Plate Count Agar (PCA), Mannitol Salt Agar (MSA), de Mann Rogossa and sharp (MRS) agar for the counts of aerobic mesophilic bacteria, staphylococci, and lactic acid bacteria (LAB). (All media used in the study were from Oxoid unless mentioned). The PCA and MSA plates were incubated under aerobic conditions for 24-48 hours at 30 to 32°C. The MRS plates were incubated at 30 to 32 °C for 24-48 hours under anaerobic conditions using anaerobic jar (BBL, Gaspack Anaerobic system). After colony counting, 10 to 15 colonies were picked randomly from MRS plates for further purification and characterization of lactic acid bacteria. The colonies of LAB were re-transferred into 5ml MRS Broth (Oxoid) and purified by repeated plating on MRS agar. The pure cultures of LAB were stored on MRS agar slant at 2°C for further characterization. **Identification of Isolates:**

The pure cultures of LAB were characterized for cell morphology, Gram reaction, catalase production, and carbohydrate utilization following standard microbiological methods (Harrigan, and McCance, 1976). Gram reaction of isolates was tested by KOH test using 3 % KOH (Gergerson, 1978). The production of catalase enzyme was determined by flooding young culture (grown overnight) with 3% solution of H₂O₂. Furthermore, the staphylococci and yeasts were identified to group level based on culture characteristics on solid media and microscopy.

pH determination. The pH of samples were measured using digital pH meter (NIG. 333, Naina solar LTD, India), after homogenizing 10ml of the sample in 90ml of distilled water.

Antibiotic Susceptibility Test: The LAB isolates were tested for sensitivity to six different antibiotics (Oxoid Ltd, UK) following the modified standard Kirby – Bauer procedure as used by Rojo-Bezares *et al.*, (2006) on MRS agar (pH 7.4±0.2). The antibiotics used in this study were selected on the basis of their mechanisms of action, targeting different sites or processes although duplicates were included in some cases. Accordingly, the test was done following the disc diffusion technique using: Pencillin G (Pen, 10µg), Erythromycin, (Ery, 15 µg), Methicillin (Met, 5 µg), tetracycline (Tet, 30 µg), Amikacin (Ak, 10 µg), and Norfloxacin (Nx, 30 µg). For quality control of the antibiotics used during the study, sensitive reference strains including *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922 were used. (The reference strains were kindly obtained from Ethiopian Health and Nutrition Research Institute).

MRS agar plates (Oxoid) were swabbed with standardized culture suspensions (0.5 MacFarland standard, equivalent to cell

density of ca:10⁸cfu/ml) using a sterile cotton swab. The pre-determined antibiotic discs were dispensed onto the surface of the inoculated agar plate. After 36 hours of incubation at 30 to 32°C under anaerobic condition (BBL, anaerobic GasPack System), inhibition zone diameter was measured. The isolates were classified as sensitive or resistant following the cut-off points given by the manufacturer (NCCLS, 2002).

Data Analysis

Data was analyzed using SPSS soft ware version 16 (SPSS Inc., Chicago, IL, USA). Coefficient of variation was calculated for the significances of differences within samples and ANOVA was employed for significances of differences between mean counts of microbial groups.

Ethical consideration

The study was conducted following formal research ethics whereby permission was obtained from the Department of Biology, Jimma University, and with the consent of owners of the food establishments’.

RESULTS

Ergo samples were found dominated by both aerobic mesophilic bacteria (AMB) and lactic acid bacteria (LAB) with mean microbial counts greater than 9 log CFU/ml in each cases (Figure.1). Relatively, the mean counts of staphylococci were the lowest (6 Log CFU/ml) although the counts of yeasts (8 Log CFU/ml) were almost comparable to that of LAB and AMB. The mean counts of all the microbial groups vary significantly within samples (CV> 10%) and the variability was the highest within counts of staphylococci (CV = 50%).

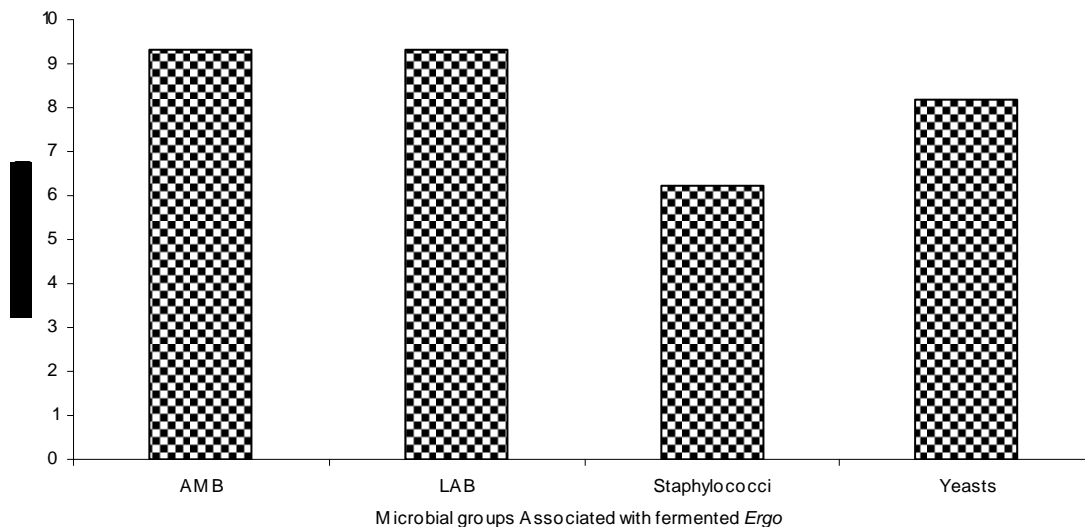


Figure 1 Major microbial composition of *Ergo* samples, Jimma town, 2009
Where, AMB= Aerobic Mesophilic Bacteria, LAB = Lactic Acid Bacteria

The mean pH of the *Ergo* samples was 4.34 ± 0.06 (Data not shown) indicating that most of the fermented milk products were acidic at time of sampling.

All the isolates characterized from MRS agar plates were found to be Gram-positive and catalase-negative, displaying the characteristic features of typical lactic acid

bacteria. Among the LAB, lactobacilli were the frequently isolated groups from all the ergo samples analyzed for its microbiology.

All strains of LAB isolated from *Ergo* were sensitive to Penicillin G and Erythromycin,

the inhibitors of cell wall synthesis in bacteria (Table 1). Similarly, 98.2% and 89.5% of the isolates were also sensitive to tetracycline and amikacin, respectively. The highest resistance was observed against Methicillin (89.5%) and norfloxacin (80.7%).

Table 1: Antibiotic sensitivity/resistance properties of some LAB isolated from Ergo, Jimma, 2009

S.NO	Antibiotics	No. of Sensitive Isolates	% of sensitive Isolates
1	Penicillin G	57	100
2	Tetracycline	56	98.2
3	Amikacin	51	89.5
4	Norfloxacin	11	19.3
5	Erythromycin	57	100
6	Methicillin	6	10.5

A total of three multiple drug resistance (MDR) patterns, resistance to two or more antibiotics, were observed within the LAB isolates (Table 2). More than half of the isolates (54.38%) were resistant to both Norfloxacin and Methicillin (Nx/Met), followed by resistance to only methicillin

(17.54%) and only Norfloxacin (15.79%). Only one isolate developed multiple drug resistance to four antimicrobials (Tet/Ak/Nx/Met) (resistance to tetracycline, amikacin, norfloxacin and methicillin simultaneously).

Table 2: Multiple drug resistance patterns of some LAB isolated from *Ergo*, Jimma, 2009

No. of resistance	Resistance pattern, including MDR	No. of Resistant Isolates	Percentage (%)
1	Nx	9	15.79
1	Met	10	17.54
2	Nx/Met	31	54.38
3	Ak/Nx/Met	6	10.52
4	Tet/Ak/Nx/Met	1	1.75

Where, Nx = Norfloxacin, Met = Methicillin, Ak = Amikacin, Tet = Tetracycline, MDR= Multiple drug resistance.

DISCUSSION

In Ethiopia, a considerable proportion of milk is consumed in a fermented state as *Ergo* or 'Ittitu'. The fermentation is usually natural, with no defined starter cultures used to initiate the fermentation processes

(Mogessie Ashenafi, 2002). As a result, the microbial load of fermented milk samples, including *Ergo*, could vary from sample to sample based on the microbial load and types of microbes in the original raw milk. The significantly high variation (CV>10%)

observed in mean counts within the ergo samples could be accounted to the dependence on natural inoculum and poor hygienic practice. The extreme variability in counts of staphylococci within samples is an indication of post production contamination.

Microbiologically, the frequently encountered microbial groups are aerobic mesophilic bacteria, LAB, staphylococci and yeasts. The microbial load observed in this study was comparable to the counts of LAB, and yeasts reported by Kassaye *et al.* (1991), although much lower than the count of AMB (10^{12} cfu/gm) of the earlier report. Usually, the counts of yeasts could rise from undetectable levels at the start of fermentation and reaches counts as high as $5 \log$ cfu/ml. This was in agreement with our observation during the study. Among the LAB that dominates the fermentation of milk are *Lactococcus* and/or *Lactobacillus* (Almaz Gonfa *et al.*, 1999). *Lactobacillus species* were the only LAB observed exclusively in the present study. Lactobacilli usually dominate the microbial succession during milk fermentation with rise in acidity. LAB dominates the fermentation of dairy product, and ergo is not exceptional. During the fermentation of milk, usually the *Streptococcus* initiate the fermentation processes and lowers down the pH to the range that favours the proliferation of lactobacilli (Jay, 2000). Under conditions of moderate fermentation and absence of higher proteolysis, which encourages the growth of pathogenic microbes, the lactobacilli could dominate the lactic flora of fermented milk. Thus, the high frequency of lactobacilli in the analysed ergo samples is a good indication of the fact the fermented ergo samples were not over adulterated and still did not pave the way for spoilage bacteria and fungi.

The mean pH observed in this study (pH = 4.34) was comparable, with relatively lower acidity, to mean pH of Ergo samples (pH = 3.65) analyzed from Borana, South Ethiopia (Kassaye *et al.*, 1991). Acidity of a product is among the factors that determine the microbial ecology of a given food product. Being acid tolerant and micro-aerophilic in its nature, part of the microbes counted as AMB could be LAB. Although members of the family Enterobacteriaceae are also among the common microbes included under AMB, most of the Enterobacteriaceae are less tolerant to acidity (in our case, pH= 4.34 ± 0.06) and are not expected in fermented dairy products. Thus, LAB and yeast are microbes responsible for the fermentation of our ergo samples. Staphylococci are hardy type microbial groups with better tolerance to salt, acidity and low water activity (Jay, 2000). Such tolerance has contributed to the better survival of staphylococci in the ergo samples. From safety points of view, the mean counts of 10^6 cfu/ml (CV.10%) needs further consideration as the mean count is within the ranges of enterotoxin production (Jay, 2000).

Fermented milk is known to be more stable and advantageous than fresh milk (Mogessie Ashenafi, 2002) as the fermentation process extends the shelf life of the raw milk besides improving the microbial quality and safety of the final product. Fermented milks have also been prescribed for curing disorders of the stomach, intestine and other related ailments (Fernandez *et al.*, 1987). The safety of our fermented ergo samples from potentially pathogenic gram-negative microbes, with counts below detectable levels in all samples, is an indication of the above facts related to fermented milk.

Although high proportions of our isolates were resistant to Norfloxacin and methicillin, none of them displayed resistance towards penicillin G and erythromycin. This observation was in contrary to report by Gfeller *et al.* (2003). Gfeller *et al.* (2003) reported the presence of potentially transferable genes conferring resistance to one or more of the antibiotics including penicillin, erythromycin, amoxicillin and tetracycline in several members of LAB. Thus, the absence of any strain resistant to many of these antibiotics among our isolates was an indication that these populations neither possessed nor acquired the resistance gene so far. Resistance to methicillin could be an intrinsic resistance, a naturally owned resistance.

Intrinsic resistance of lactic acid bacteria to many antibiotics may be considered as advantageous for those isolates with probiotic potential. Such resistance could be helpful for sustainable utilization of the strains in human intestine to maintain the natural balance of intestinal microflora during antibiotic therapy (Ketema Bacha *et al.*, 2010). However, there is the danger of transferring multiple drug resistance to pathogens in the intestinal environment. The susceptibility of our LAB isolates to the clinically important antimicrobials, on the other hand, is beneficial as it minimizes the chances of disseminating resistance genes to pathogens both in the food matrix and/or in the gastrointestinal tract. It could, thus, be concluded that our isolates are not reservoirs of transferable resistance genes for at least erythromycin and penicillin G, as all isolates were sensitive to these two antibiotics. This is particularly important, because our traditional fermented milk product, *ergo*, is also consumed without any further heat treatment; and some of the LAB isolates exhibited *in-vitro* probiotic potential (Ketema Bacha, *et al.*, 2009).

Based on the findings of this study, it could be concluded that the microbiology of ergo fermentation is dominated by both aerobic mesophilic bacteria and lactic acid bacteria. Although there was variability among samples of ergo collected from different cafes in terms of microbial load and pH, the mean pH of ergo samples (4.34) is in the acidic range. The acidity could give protection to the fermented product against contamination with potentially pathogenic microorganisms such as *Salmonella* and *Escherichia coli*. All the lactic acid bacteria isolated from fermented ergo were sensitive to Penicillin G and erythromycin. In general, the isolates of LAB were not found reservoirs for transferable resistance genes for Penicillin G and Erythromycin as indicated by the sensitivity of isolates. Therefore, LAB isolated from traditional Ethiopian fermented food could still be used for the enhancement of consumer's health with periodic monitoring of the existing level of drug resistance.

This study investigated, besides the microbial load, the antibiotic susceptibility patterns of strains of lactic acid bacteria isolated from ergo. The current data supplemented the earlier single report from Ethiopia (Ketema Bacha *et al.*, 2010). To have a clear picture of the current antibiotic sensitivity patterns of LAB associated with the traditional Ethiopian fermented foods, it calls for evaluation of the resistance pattern of LAB from other traditional fermented foods.

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