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Isolation and Identification of Lactic Acid Bacteria from Cow Milk using Biolog Microbial Identification System

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Abstract: Lactic acid bacteria are widely found in nature and are present naturally in several raw materials like milk, meat, flour and in many fermented foods. This study was done to isolate and identify Lactic Acid Bacteria from Cow milk using Biolog Microbial Identification System and conserve them. Atotal of355cow milk samples were collected from lactating cows. Lactic Acid Bacteria were isolated by dilution spread plate method and identified based on Bergey'sManual of Systematic Bacteriology.Thecarbohydrate fermentation patterns and chemical resistance tests were determined using Biolog GEN III Bacteria Identification System.Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus coryniformis subspecies torquens, Lactococcus lactis subspecies lactis, Weissela viridescens, Enterococcus durans, Enterococcus gallinarum, and Streptococcus thermophilus were were identified from these identified Lactic Acid Bacteria, Weissela species (33.51 %)and Enterococcus (30.89%) species were found to be the first dominant genus and the second dominant genus, respectively. It can be generalised that using a combination of media in addition to Biolog recommended media (Biolog Universal Agar) is good to increase the spectrum of Lactic Acid Bacteria isolates.

Key words: Biolog microbial identification system, Cow milk,Lactic acid bacteria.

1. INTRODUCTION

Lactic acid bacteria comprise an ecologically diverse group of microorganismsunited by formation of lactic acid as the primary metabolite of sugarmetabolism. These bacteria utilize sugars by either homo-or hetero-fermentativepathways. The term Lactic Acid Bacteria (LAB) was gradually accepted in the beginning of the 20th century[1]. Other terms as "milk souring" and "lactic acid producing" bacteria had previously been used for the same bacteria causing a slight confusion.

Many of them are generally recognized as safe (GRAS) or qualified presumption of safety (QPS). These bacteria are widely found in nature, including the gastrointestinal and urogenital tracts of humans and animals, and are present naturally in several raw materials like milk, meat, flour and in many fermented foods such as dairy products like cheeses, whey and yoghurts, and with cereal-, vegetable- and meat-based fermented foods, either as intentionally added starters or due to their natural presence leading to spontaneous fermentation [2, 3, 4]

Korhonen[4] also mentioned that LAB in addition to be found in environments rich in carbohydrates, they have complex nutritional requirements for aminoacids, peptides, fatty acid esters, salts, nucleic acid derivatives and vitamins. He further explained that even though they have complex nutritional requirements due to their lack of many biosynthetic pathways, they are on the other hand found in a wide range of different environmental niches due to their good capacity for adaptation.

Lactic acid bacteria (LAB) form part of the Gram-positive bacteria with "low" (55 mol%) G+C in their DNA. They belong to class III (*Bacilli*) of the phylum*Firmicutes*,which comprisesthree classes, the other two being *Clostridia* (class I) and *Mollicutes*(class II).Within class III, LAB are represented in order II, the *Lactobacillales*. They may be considered as a "rapidly expanding" group of bacteria,presently with six families and about 40 genera. For some of these genera the familyposition has not been finalized definitively. Some new genera such as *Atopobacter*and *Bavariicoccus*, for example, appear to belong to the family *Carnobacteriaceae*[5]. The wide range of the six families gives an impression of the diversity withinLAB:Aerococcaceae (with seven genera), Carnobacteriaceae (with 16 genera), Enterococcaceae (with seven genera), Lactobacillaceae (with three genera), Leuconostoccaceae (with four genera) and Streptococcaceae (with three genera).

Orla-Jensen'[6] publication of a monograph about lactic acid bacteria had great impact on the systematic of LAB. His classification of LAB genera was based on morphology, mode of glucose fermentation, growth at certain temperatures, and range of sugar utilization. Even though the taxonomy has been revised since then, characters used by Orla-Jensen are still very important in current classification of LAB. Lactic acid bacteria constitute a group of bacteria that have morphological, metabolic and physiological similarities, and they are also relatively closely related phylogenetically[7].

The generaldescription of the bacteria included in the group is gram-positive, acid-tolerant and non-spore forming cocci and rods, which produce lactic acid as the major end product during the fermentation ofcarbohydrates, catalase-negative, and anaerobic bacteria with low G+C. The boundaries of the group havebeen subject to some controversy, but historically the genera *Lactobacillus, Leuconostoc,Pediococcus,* and *Streptococcus* form the core of the group. Taxonomic revisionsof these genera and the description of new genera mean that LAB could, in theirbroad physiological definition, comprise around 20 genera. However, from a practical,food-technology point of view, the following genera are considered the principal LAB:*Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc,Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissel*[2, 3, 4, 8,9].16S rRNA gene sequence analysis in the past showed that only little correlation exists between the traditional classification and the phylogenetic relatedness of lactic acid acteria. As a result of these studies, it has been proposed to subdivide some of the genera found in the traditional classification into new phylogenetic groups[3]. And, currently many more genera are included in the group of Lactic Acid Bacteria and reaches to a total of 40 genera. Even though recent taxonomic revisions have proposed several new genera and comprises the above mentioned remaining group, the common agreement is that there is a core group of LAB consisting of four genera; *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus*[7, 9].

It is well known that lactic acid bacteria (LAB) have had a significant impacton human culture, traditions, and well-beingthroughout human history. In modern times, their economicimportance has increased tremendously as a result of the industrialization of foodbio-transformations. In particular, they have a key function in the development ofthe sensory and safety features of fermented food products. Moreover, in the development of newapplications such as probiotic foods, pharmaceutical preparations, and live vaccines,the need for robust LAB is even more important. As a result of the association of LAB with fermented foods and with human andanimal health, LABenjoy increasing importance and consideration from scientists,industries, and consumers in modern society[5]. LAB have a great potential to play a role in food preservation. Nowadays, consumers favor food with few chemical preservatives. As a result, there isincreased interest in the preservation through LAB because of their safe association withhuman fermented foods. Several metabolic products produced by these bacteria haveantimicrobial effects, including organic acids, fatty acids, hydrogen peroxide anddiacetyl. However, attention has focused on the ability of LAB to produce specificproteinaceous substances, bacteriocins that inhibit the growth of pathogens, such as*Listeria, Clostridium, Staphylococcus, Bacillus* spp. and *Enterococcus* spp., thereby; they enhance the shelf life of the food[10].

Lactic acid bacteria have contributed inincreased volume of fermented foods worldwide especially in foods containing probioticsor health promoting bacteria.Probiotics are live microbial, dietary supplements or food ingredients that have abeneficial effect on the host by influencing the composition and metabolic activity of theflora of the gastrointestinal tract[11].Tsakalidou and Papadimitriou[5] (2011) expressed, in their book entitled "*Stress Responses of LacticAcid Bacteria*", that in terms of total biomass, enormousquantities of LAB are being consumed in our daily diet. Traditionally, this is primarilyrelated to fermented foods, but an increasing amount of LAB biomass can nowalso be allocated to functional foods, of which the probiotics comprise the largestand most rapidly growing segment of the market. The International Dairy Federation(IDF) reported the average annual consumption of fermented milk products to be22 kg per capita in Europe, which amounts to around 8.5billion kg of fermented milk per year. This figure does not take into account theLAB used in nondairy food fermentations (vegetables, meat, legumes, etc.) or probioticproducts containing selected strains with beneficial health features. Thus, theactual amount of lactic acid bacterial biomass would be far greater.Therefore, the exploitation ofLAB in industrial processes should become more strongly target-oriented and"streamlined".Probiotic strains should typically be acid resistant and bile tolerant,they should survive gastrointestinal conditions, and they should be able to bindto epithelial cells or the gastrointestinal mucus in order to colonize. Thus, despite selection for robust strains, there is also still a need for thedevelopment of methods that protect the bacteria and increase their viability duringprocessing and storage.

Lactic acid bacteria (LAB) predominate in microflora ofmilk and milk products, many species are involved in the daily manufacturing of dairyproducts[12]. The lactic acid bacteria used in the dairy fermentation can roughly be divided in to twogroups based on their growth optimum. Mesophilic lactic acid bacteria have anoptimum growth temperature between 20^{O}C and 30^{O}C and the thermophilic LAB have theiroptimum between 30^{O}C and 45^{O}C. It is not surprising to discover that traditionalfermented products from sub-tropical countries harbor mainly thermophilic lactic acidbacteria, whereas the products with mesophilic bacteria originated from western andnorthern European countries[13]. The thermophilic lactic acid bacteriaare best known as a starter for fermented milks.The LAB used in commercial starter culture possesses numerous metaboliccharacteristics such as acidification activity, proteolytic activity, synthesis of bacteriocin,resistance to bacteriophages and production of exopolysaccharaides are strain dependent.All of these important activities contribute to

the flavor, texture and frequently thenutritional attributes of the product[12].

Lactic acid bacteria, as mentioned earlier in this paper, have an important role in the inhibition of food-born pathogenic andspoilage microorganisms with antimicrobial metabolites, including lactic acid, aceticacid, and other organic acids, hydrogen peroxide, bacteriocins and bacteriocin-likesubstances.Bacteriocins produced by LAB are the subject of intense research because of theirantibacterial activity against food-borne bacteria. Bacteriocins consistof a biologically active protein moiety, have a bactericidal mode of action and attach tospecific cell receptor. They are heterogeneous group of bacterial antagonists thatvary considerably in molecular weight, biochemical property, and range of sensitive host andmode of action. Both Gram-negative and Gram-positive bacteria produce them. TheGram-negative bacteriocins are colicin, which are produced by strain of *E.coli*.Most of the Gram-positive bacteriocins are membrane active compounds that increase thepermeability of the cytoplasmic membrane[14]. They show much broaderspectrum of bactericidal activity than the colicins. Many bacteriocins of LAB are safe andeffective natural inhibitors of pathogenic and food spoilage bacteria in various foods.Nisin is the classic example; it prevents C*lostridial*spoilage of processed and naturalcheeses, inhibits the growth of some psychrotrophic bacteria in cottage cheeses, extendsthe shelf life of milk in warm countries, prevents the growth of spoilage *Lactobacilli* inbeer and wine fermentations and provides additional protection against *Bacillus* and*Clostridial*spores in canned foods. Nisin is a permitted food additive in more than 50countries including the USA and Europe under the trade name Nisaplin[15].

As tried to be explained about the importance of LAB in the above paragraphs, LAB do have tremendous advantages to humans. Taking these economic importance and health benefits of LAB to humans and the different agroecological zones of our counry, and various livestock breed types, this study was developed to collect Cow milk from areas vicinity to Addis Ababa and isolate and identify Lactic Acid Bacteria (LAB) with the intention of characterizing and preserving LAB for further utilization and research for the Ethiopian community.

2. MATERIALS AND METHODS

2.1 Study time

Sample collection and LAB isolation, identification and preservation were carried out from December to April 2014/15 G.C. Samples were collected from nearby areas of Addis Ababa.

2.2 Study areas

A. Cow milk sample collection areas

Samples were collected from milk production areas located nearby Addis Ababa. The milk production system in nearby areas of Addis Ababa is classified as peri-urban and urban dairy productionsystems. These dairy production systems may beidentified as small urban/peri-urban systems raising crossbred or both crossbredand local cattle having access to milk collection centres or cooperatives. The main zones and woredas of the regions where sample collection carried out are listed in table 1 below and depicted in figure 1. GPS data of the sample collection areas were also recorded.

B. Area of laboratory isolation and identification of LAB

Sample processing, laboratory isolation, identification and preservation of bacteria were carried out in Microbiology Laboratory of EBI.

2.3 Study population and sampling techniques

The study included lactating cows of both crossbredand local varieties. Zonal and woreda agriculture bureau were contacted to obtain the important base-line data about the potential milk production of dairy farmers living in each *kebeles* (the smallest administrative divisions in each region of Ethiopia) and get guidance during sample collection.

Table 1: Region, woredas and specific area/kebele of sample collection areas

Region	Major Woreda	Specific area/Kebele	Total samples
Oromia	Kuyu woreda	Gebre gurecha	28*5
		Olaumo	4*5
		Worabi	6*5
	Degam woreda	Alidoro	7*5
		Anajiru	2*5
		Gendeshino	12*5
		Gendewletu	12*5
Total			355

**merged samples in five*

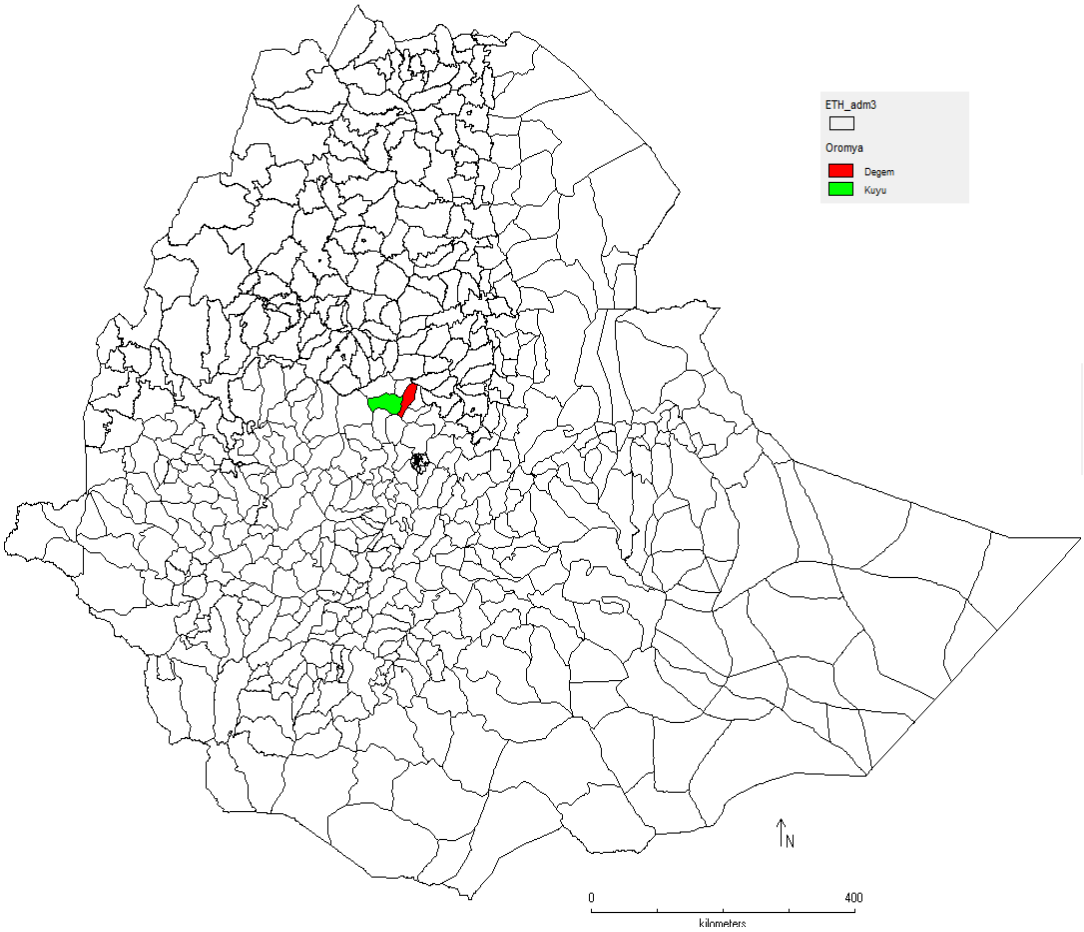

Fig. 1 Map of sample collection areas

Based on this base-line data, rural*Kebeles*found in two *Woredas* which have good milk productionpotentials were selected using purposive sampling. Seven *kebeles* were included in the study from two *woredas*(Larger administrative division in Ethiopia and comprises around 20-25 *kebeles*). And, farmers' households who own cows from each rural *Kebeles*were selected randomly. Milk samples were collected on availability basis from the randomly selected households. Households which have one or more lactating cows were taken as milk sources. Sufficient number of farmers' households were included using systematicrandom sampling methods to meet our total sample number requirement.

2.4 Sample size and type

From the randomly selected dairy farmers' households, a total of 355 samples (20 ml - 50 ml each) were collected using sterile plastic test tubes. And then, the 355 cow milk samples were merged into 71 composite samples.

2.5 Sample collection and transportation methods

I. Sample collection method

Raw cow milk samples were collected after the udder was washed with antiseptic solution, wiped dry with clean cloth and then disinfected with cotton ball dampened with 70% alcohol. The foremilk were discarded and 20 ml-50 ml of pooled milk were collected (about 5-15 ml from each quarter).

II. Sample transportation method

Samples were transported to EBI Microbiology Laboratoryunder refrigeration temperature (4 ^{O}C) in an icebox. Sample analysis was performed shortly after arrival of the samples to the laboratory. Aseptic techniques were followed during sample collection and sample transportation as described by FDA[16] and HPA[17].

2.6.Cow milk samplesprocessing

The 355 cow milk samples were merged into 71 test tubes. Samples collected from the same *Kebeles* were merged only if they are similar in sources and pH value. Samples were analyzed by the dilution spread plate method.About 10 mlof milk sub-samples were asepticallyhomogenized separately in 100 ml of sterilesaline solution (0.85%) or distilled waterfor 2 minutes usingvortex mixer. Appropriate serial dilutions(up to 10^{-8}) were made by transferring 1ml ofhomogenate into 9ml of sterile salinesolution[18].

2.7 Lactic acid bacteria isolation and identification

I. Isolation of LAB from cow's raw milk

About 0.1 mlof appropriate dilution(10^{-4}, 10^{-5}, and 10^{-6}) of the homogenized sample were surface plated in triplicates

onpre-dried surface of de Man, Rogosa and Sharpe (MRS), Elliker agar and BUA forthe counts of lactic acid bacteria(LAB). The MSA, Elliker agar and BUA plates were incubated at 30 to 32^{O}C for 24-48 hours under anaerobic conditions using anaerobic jar. The pH of the media was adjusted according to the measured pH values of original samples. The plates with colony forming units (CFU) ranging from 30 and 300 were selected fore numeration. After colony counting, 10 to 15 colonies were picked randomly from MSA, Elliker agar and BUA plates forfurther purification and characterization oflactic acid bacteria. The suspected colonies of LAB were purified by repeated streaking on MSA, Elliker agar and BUA agar. The pure cultures of LAB were stored in 10% glycerol at -20^{O}C for further haracterization[18]. MRS was primarily used for isolating *Lactobacilli,Pediococcus* and *Leuconostoc*. Eliker agar was employed for isolating lactic streptococci (*Lactococcus* and *Streptococcus*) and *Lactobacilli*. BUA (Biolog Universal Anaerobic agar) was used for growing and isolating anaerobic lactic acid bacteria.

II. Identification of lactic acid bacteriafrom cow's raw milk

Identification of LAB were performedaccording to their morphological, cultural,physiological and biochemical characteristicsdescribed in the Bergey'sManual of Systematic Bacteriology[3].Preliminary identification of LAB isolates were made by examination for cell morphology usingoptical microscopy, Gram staining, catalase and oxidase activity, and colony morphology[18]. Gram-positive, catalase and oxidase-negative, cocci or rod-shaped, non spore forming isolates were considered as presumptive lactic acid bacteria. Thecarbohydrate fermentation patterns and chemical resistance tests were determined using Biolog GEN III Bacteria Identification System Kit.Theisolated bacteria were identified to species level using BIOLOG GEN III (Omnilog, Hayward, CA) following the manufactures' instuctions to identify strains[19].

The Biolog GEN III Micro Plate analyzes a microorganism in 94 phenotypic tests: 71 carbon sourceutilization assays and 23 chemical sensitivity assays. The test panel provides a "Phenotypic Fingerprint"of the microorganism that can beused to identify it at the species level.The plates contained 96 wells, with a dehydrated panel of necessary nutrient medium (a carbon source), biochemicals and tetrazolium violet. Tetrazolium violet is a purple formazan, a redox dye that turns purple when reduced, indicating use of the carbon source provided or resistance to inhibitory chemicals. Each plate contained a positive and negative control well.

Pure culture of bacteria isolates were grown on Biolog BUG and/or BUA agar plates in anaerobic jar at 30 ^{0}C - 32 ^{0}C for 20-24 hours.Single colonies were swabbed and suspended in inoculating fluid C. Cell suspensions (100 µl) adjusted at 90-98% transmitance were pipetted into 96 well Biolog Microplates for carbon utilization and chemical test. Panels were incubated at 30 ^{0}C - 32 ^{0}C in anaerobic jar with anero-sachet genereting gas for 20-24 hours. The mcroplates were inserted into the Biolog-Omnilog automatic system and the identification process was carried out using GEN III Biolog-Omnilog identification systemsoftware[19].

2.8 Data analysis

Data were recorded in Exell spread sheet and imported to SPSS to analyze using SPSS software version 20 (SPSS Inc., Chicago, IL, USA).Coefficient of variation was calculated forthe significances of differences with in samples and ANOVA were employed for significances of differences between mean counts of microbial groups.

3. RESULTS AND DISCUSSION

3.1 Isolation of lactic acid bacteria

A total of 364 pure colony isolates were selected, of which 110, 109 and 145 isolates were obtained from MRS, Elliker and BUA agar medium, respectively. Based on preliminary identification methods of LAB; by examination for cell morphology using optical microscopy, Gram staining, catalase activity, oxidase activity, and colony morphology, the LAB suspect isolates were screened. Gram-positive, catalase and oxidase-negative, cocci or rod-shaped, non spore forming isolates were considered as presumptive lactic acid bacteria[20]. A total of 191 LAB suspect isolates were screened. Seventy six, 65 and 50 LAB suspect isolates were selected from MRS, Elliker and BUA agar, respectively. And, these 191 LAB suspect isolates were clustered into morphological groups. Three groups from MRS, 3 groups from Elliker and 2 groups from BUA were formed, respectively. And, by taking one representative isolate from each morphological groups, a total of eight representative isolates were subjected to Biolog GEN III Bacterial Identification System.

3.2 Identification of the isolated lactic acid bacteria

I. Isolated and identified genera of lactic acid bacteria:

Five different genera of Lactic Acid Bacteria were isolated which include *Weissela* (33.51 %), *Lactobacillus* (19.9 %), *Streptococcus* (2.62 %), *Enterococcus* (30.89 %), and *Lactococcus* (13.09 %) [Table 2]. Sixity four isolates picked from MRS agar plates were found to belong to the genus *Weissela*, 7 isolates were *Lactobacillus* and 5 were *Streptococcu*s making a total of 76 lactic acid bacteria isolated on MRS agar (Table 2). Fifty nine isolates selected from Elliker agar were found to be genus *Enterococcus* and 6 isolates were *Lactobacillus* making a total of 65 lactic acid bacteria isolated on Elliker agar (Table 2). From the 50 bacteria isolated on BUA, 25 belonged to genus *Lactobacilli*, and 25 were *Lactococcus* (Table 2). This result iscomparable to Ali's[21a]result who isolated *Lactobacillus,Lactococcus*, and *Enterococcus* from cow milk. This result is also in line with the results reported by Savadogo *et al*[20]and Assefa *et al*[22].who

isolated five genera of lactic acid bacteria from *Ergo*. The LAB they isolated comprised *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus* and *Streptococcus*.

Table 2: Isolated and identified genera of lactic acid bacteria from cow milk

No.	Lactic acid bacteria genera	% of isolates
1	Weissela	33.51
2	Lactobacillus	19.9
3	Streptococcus	2.62
4	Enterococcus	30.89
5	Lactococcus	13.09

In this study, Weissela isolated on MRS agar was the dominant genus which comprised 33.51 % of the total lactic acid bacteria isolates (Table 2). Enterococcus (30.89 %) was the second dominant genus isolated in this study. Higher number of LAB were isolated on MRS (39.79%) and Elliker (34.03 %) than BUA (26.18%) agar [Table 3] which indicated that using media other than Biolog recommended media (BUA) is good to increase the spectrum of LAB isolates for Biolog Identification System.

II. Isolated and identified species of lactic acid bacteria:

The 191 isolates screened from milk samples wereidentified by using Biolog Bacteria Identification System Kit. The following lactic aid bacteria were identified after Biolog's carbon source utilization and chemical sensitivity assay was performed on Biolog GEN III Microplates: *Lactobacillus plantarum* (13.09%), *Lactobacillus rhamnosus* (3.14%), *Lactobacillus coryniformis ss torquens* (3.67%), *Lactococcus lactis* subspecies *lactis* (13.09%), *Weissela viridescens /formerly Lactobacillus viridescens/* (33.51%), *Enterococcus durans* (23.04%), *Enterococcus gallinarum* (7.85%), *and Streptococcus thermophilus* (2.62%) [Table 3 and 4]). Table 3 depicts the reading result of Biolog GEN III Omnilog plus identification softare system for the isolated LAB. The accuracy of the identified lactic acid bacteria was assesed based on the Manufacturer's interpretation criteria which indicates that one can be confident that ID #1 is accurate if the top-ranked ID choices on the list are all the same (orclosely related) genera, the SIM (similarity index value) rating of ID #1 is 0.50 or above at 16-22hours and the DIST (distance) rating of ID #1 is 0.50 orless[19].

Table 3: Biolog-Omnilog GEN III identification system's reading result of isolated LAB

Media	No.	Lactic acid bacteria	PROB	SIM	DIST	Organism type
MRS	1	*Weissela viridescens*	----	0.303	4.883	GP-Rod
	2	*Lactobacillus coryniformis ss torquens*	0.287	0.787	2.978	GP-Rod
	3	*Streptococcus thermophilus*	-----	0.279	4.670	GP-Coccus
Elliker	4	*Enterococcus gallinarum*	0.365	0.603	5.768	GP-Coccus
	5	*Enterococcus durans*	0.363	0.674	4.617	GP-Coccus
	6	*Lactobacillus rhamnosus*	----	0.356	5.172	GP-Rod
BUA	7	*Lactococcus lactis ss lactis*	0.551	0.617	5.514	GP-Coccus
	8	*Lactobacillus plantarum*	0.395	0.624	5.489	GP-Rod

PROB*: Probability, SIM: Similarity, DIST: Distance, MRS: de Man Regosa and Sharpe, BUA: Biolog Universal Anaerobe.*

Ali[21a]isolated *Lactobacillus plantarum* (21.74 %) from cow milk collected in Khartoum, Sudan which is in agreement with the result of this study.But, he isolated greater percentage of *Lactobacillus rhamnosus* (17.39 %) than this study. Ashmaig*et al.*isolated *Lactobacillus rhamnosus* from *gariss*, Sudanese traditional fermented camel milk, in 4.17 % which is in line with the 3.14 % isolation rate in this study. *Lactococcus lactis* subspecies *lactis* was found to be 13.09 % which is comparable with the isolation rate of 21.7 % and 17.8% observed by Ali[21a]from cow milk and Seifu *et al*.[24]from camel milk, respectively. Rabha *et al*.[25]isolated *Streptococcus thermophilus* in 10 % from cow's raw milk which is moderately comparable with the result of this study. Mezaini*et al.*[26]also isolated *Streptococcus thermophilus* in 10 % from *raib*, Algerian traditional fermented milk. *Enterococcus durans* was isolated from 23.04 % of cow milk samples in this study which is much greater than the isolarion rate reported by Ali[21b]which was only 0.43 %.

III. Morphological, biochemical and physiological characteristics of the isolated LAB

Table 5 summarizes the morphological, biochemical and physiological tests of eight representative LAB isolates determined by Biolog Bacteria Identification System and conventional microbiological tests.

Table 4: Isolated and identified species of lactic acid bacteria from cow milk

Isolation media	Cluster group	Species	No. of isolates	% of total isolates
MRS	1	*Weissela viridescens*	64	33.51
	2	*Lactobacillus coryniformis ss torquens*	7	3.67
	3	*Streptococcus thermophilus*	5	2.62
Sub-total			76	39.79
Eliker	4	*Lactobacillus rhamnosus*	6	3.14
	5	*Enterococcus durans*	44	23.04
	6	*Enterococcus gallinarum*	15	7.85
Sub-total			65	34.03
BUA	7	*Lactococcus lactis ss lactis*	25	13.09
	8	*Lactobacillus plantarum*	25	13.09
Sub total			50	26.18
Total			191	100

All strainstested are Gram positive, catalase and oxidase negative.However, they were different in cell and colony morphology. MRS53, EL10, EL31 and BUA19 isolates showed a coccal morphology, whereas the rest four representative strains showed a rod-shaped morphology.

Growth at pH 5 and 6

EL10 and BUA19 isolates were able to grow at pH 5 and 6. But, MRS53 isolate was neither able to grow at pH 5 nor pH 6. MRS3 and EL31 isolates were able to grow at pH 6 but not at pH 5. The growth of MRS7, EL45 and BUA27 isolates at pH 5 and 6 was uncertain or found at borderline.

Growth in different percent of NaCl solution

EL10, EL31 and BUA19 isolates were able to grow in 1% NaCl solution but neither of the isolates were able to grow in 8 % NaCl solution. Isolates MRS53, EL45 and BUA27 were not able to grow in 4 % NaCl solution as indicated in table 5.

Table 5: Morphological, biochemical, physiological characteristics of the isolated LAB

Characteristics (Tests)		Lactic Acid Bacteria species							
		MRS3 (Cluster group 1)	MRS7 (Cluster group 2)	MRS53 (Cluster group 3)	EL10 (Cluster group 4)	EL31 (Cluster group 5)	EL45 (Cluster group 6)	BUA19 (Cluster group 7)	BUA27 (Cluster group 8)
Biochemical									
i. Gram stain		GP	GP	GP	GP	GP	GP	GP	GP
ii. Cell morphology		Rod	Rod	Coccus	Coccus	Coccus	Rod	Coccus	Rod
iii. Catalase test		-	-	-	-	-	-	-	-
iv. Oxidase test		-	-	-	-	-	-	-	-
Physiological									
i. Growth at pH	5	{x}	{x}	x	<x>	{x}	{x}	<x>	{x}
	6	<x>	{x}	x	<x>	<x>	{x}	<x>	{x}
ii. Growth in NaCl	1%	{x}	<x-	x	<x>	<x>	<x-	<x>	{x}
	4%	{x}	{x}	x	{x}	{x}	x	{x}	x
	8%	x	x	x	{x}	{x}	x	x	x

Key: MRS3: *Weissela viridescens;* MRS7: *Lactobacillus coryniformis ss torquens;* MRS53: *Streptococcus thermophilus,* EL10: *Enterococcus gallinarum,* EL31: *Enterococcus durans,* EL45: *Lactobacillus rhamnosus,* BUA19: *Lactococcus lactis ss lactis,* BUA27: *Lactobacillus plantarum,*<x>: positive, x: negative, <x-:mismatched positive, x+: mismatched negative, {x}: borderline, -x: less than A1 well

Carbon utilization profiles of the isolated lactic acid bacteria

Table 5 summarizes major carbon utilization profiles of the isolated lactic acid bacteria determined by Biolog Bacteria Identification System. All the isolated Lactic acid bacteria fermented one or another carbohydrate sources but rhamanose,raffinose and arabitol. Lactic acid bacteria group that fermented only glucose, mannose, and fructose was

identified as *Lactobacillus coryniformis ss torquens.* Lactobacillus isolates that fermented trehalose, lactose, salicin, glucose, mannose, fructose galactose, and mannitol were classified as *Lactobacillus rhamnosus.* Lactobacillus isolates which fermented maltose, sucrose, glucose, mannose, mannitol but showed weak fermentation for trehalose, salicin, fructose, sorbitol, and arabitol were classified as *Lactobacillus plantarum.*

Table 6: Major carbon source utilization assay results of the isolated lactic acid bacteria

Type of carbohydrate	Lactic Acid Bacteria species							
	MRS3 (Cluster group1)	MRS7 (Cluster group2)	MRS53 (Cluster group 3)	EL10 (Cluster group4)	EL31 (Cluster group5)	EL45 (Cluster group6)	BUA19 (Cluster group 7)	BUA27 (Cluster group 8)
Maltose	<x>	x	x	<x>	<x>	x	<x>	<x>
Trehalose	<x>	x	x	<x>	< x -	<x>	<x>	{x}
Sucrose	{x}	x	<x>	x +	x	x	< x -	<x>
Raffinose	x	x	< x -	x	x	x	{ +	x
Lactose	{x}	x	<x>	<x>	<x>	<x>	<x>	< x -
Salicin	<x>	x	x	<x>	<x>	<x>	<x>	{x}
Glucose	<x>	<x>	<x>	<x>	<x>	<x>	<x>	<x>
Mannose	<x>	<x>	< x -	<x>	<x>	<x>	<x>	<x>
Fructose	<x>	<x>	<x>	<x>	<x>	<x>	<x>	{x}
Galactose	<x>	< x -	{ x -	<x>	<x>	<x>	<x>	x
Rhamanose	x	x	x	x	x	x +	x	x
Sorbitol	{x}	x	{x}	x	{x}	< x -	< x>	{x}
Mannitol	<x>	x	{x}	x +	x	<x>	< x>	<x>
Arabitol	x	x	x	x	x	x	x	{x}

Key: MRS3: *Weissela viridescens;* MRS7: *Lactobacillus coryniformis ss torquens;* MRS53: *Streptococcus thermophilus,* EL10: *Enterococcus gallinarum,* EL31: *Enterococcus durans,* EL45: *Lactobacillus rhamnosus,* BUA19: *Lactococcus lactis ss lactis,* BUA27: *Lactobacillus plantarum,*<x>: positive, x: negative, <x-:mismatched positive, x+: mismatched negative, {x}: borderline, -x: less than A1 well

Lactic acid bacteria group that fermented maltose, trehalose, lactose, salicin, glucose, mannose, fructose, galactose,sorbitol, mannitol but cannot ferment rhamanose, arabitol and raffinose was identified as Lactococcus lactis subspecies lactis. This result is in agreement withSeifu et al. [24] and Cheriguene et al. [27] who reported in their study that Lactococcus lactis subspecies lactis was unable to ferment raffinose, sorbitol and arabinose. Lactic acid bacteria group that fermented maltose, trehalose, lactose, salicin, glucose, mannose, fructose, and galactose was identified as Enterococcus gallinarum. Enteroccoccus durans fermented all carbohydrate sources fermented by Enterococcus gallinarum excepet trehalose and also showed weak fermentation for sorbitol.Streptococcus thermophilus isolates were able to ferment sucrose, lactose, glucose, and fructose. But, they were not able to ferment maltose, trehalose, salicin, rhamanose, and arabitol.

4. CONCLUSION

In the present study, Weissela species was found to be the dominant genus and comprised of 33.51 % of the total lactic acid bacteria isolates. Enterococcus (30.89% of the total lactic acid bacteria isolates) was the second dominant genus. It can be generalised that using a combination of media in addition to Biolog recommended media (BUA) is good to increase the spectrum of Lactic Acid Bacteria isolates.

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