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Exploring antimicrobial activity of *Lactobacillus* spp. (probiotics) isolated from raw cow's milk against *Staphylococcus aureus* causing bovine mastitis

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ABSTRACT

Bovine mastitis is a costly disease in the dairy farms globally. The control of such disease is generally based on the prevention by the strict hygienic measures during milking. Other approaches include vaccination and the application of antibiotics. Regardless to these procedures, mastitis is not entirely under the control, thus increasing the need for alternative tactics. This study was conducted to isolate and identify lactic acid bacteria (LAB) from fresh cow's milk which possess antibacterial activity that could be used for mastitis control. 146 isolates were recognized as (LAB) from105 milk tanks samples after being cultured anaerobically on de Man, Rogosa and Sharpe (MRS) agar plates for 48 hours at 37 ^{o}C and identified by general bacteriological investigation. Afterwards, 24 isolates were identified to belong to genus *Lactobacillus* using polymerase chain reaction (PCR), and for species level recognition MALDI-TOF MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry) was used resulted in : *L. fermentum* (5), *L. brevis* (3), *L. plantarum* (4), *L. paracasei* (2), *L. rhamnosus* (3), *L. pentosus* (2), *L. casei* (3), *L. raffinolactis* (1) and *L. mesenteroids* (1). The antimicrobial activity of these strains against one of the major mastitis pathogens, *S. aureus*, was detected by the agar well diffusion assay and the modified double layer method , where *L. casei, L. fermentum* and *L. plantarum* possess the most inhibiting effect besides they have no hemolytic nor gelatin liquefaction activity when their safety profiles were evaluated. The result of the antibiotic susceptibility test revealed that these isolates were resistant to vancomycin (VA), neomycin (N) and gentamycin (CN). On the other hands, they were highly sensitive to amoxicillin clavulanic acid (AMC), Levofloxacin (LEV), tetracycline (TE) and penicillin (P). The study suggests that *L. casei, L. fermentum* and *L. plantarum* are perfect candidates to be used as probiotics to help in preventing and controlling bovine mastitis caused by *Staphylococcus aureus* as they were proven to be safe and have antimicrobial activity against the organism.

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INTRODUCTION

For many years probiotics have been studied for their beneficial effects on human and animal health **(Reid *et al.*, 2006 and Behnsen *et al.*, 2013).** Probiotics contribute to preserve the stability of natural microbiota of the host niche by competing with the pathogen for tissue colonization, controlling virulence expression or stimulating the innate immune system **(Sengupta *et al.*, 2013 and Even *et al.*, 2014)**. With few exceptions, most probiotic products currently available contain lactic acid bacteria, which mainly belong to the genera *Lactobacillus* and *Bifidobacterium*. The scientific papers published in major microbiological and nutrition journals recommend evidence of the beneficial effects of probiotics **(Lourens-Hattingh and Viljoen, 2001).**

Nowadays the administration of probiotics is considered as an alternative method for the prevention and treatment of infections. It is believed that preventive treatment with probiotic product could decrease the usage of antibiotics. Probiotics do not cause negative impact on gut microflora **(Serikbayeva *et al.*, 2005** and **Reid *et al.*, 2006)** and provide 'healthy bacteria' include *Lactobacillus* strains, *Bifidobacterium*, and *Enterococcus faecium* **(Shirley and Jean 2010)**. In animal production sector, probiotic bacteria have been used in the feeds or drinking water of cattle, poultry, pigs, and fish, to improve their performance **(Dowarah, R. *et al.*, 2017 and Vieco-Saiz, N. *et al.*, 2019).**

Also, the use of probiotics has gained attention to combat bovine mastitis (an inflammatory condition of the mammary gland) which usually resulted from bacterial infection causing massive economic losses in the dairy farms and dairy industries **(Contreras and Rodriguez, 2011 and Le Marechal *et al.*, 2011).** Lactic acid bacteria (LAB) can afford protection against mastitis when they are used in diets , teat dip, and intramammary inoculation due to their strong immunomodulatory effect (**Rainard and Foucras, 2018)**. LAB can form a protective biofilm in the udder which inhibits the growth of pathogens and prevent mastitis (**Wallis *et al.*, 2018).** The bovine mammary microbiota was investigated to identify microorganisms with inhibitory properties against mastitis pathogens **(Espeche *et al.*, 2012)**.

The world health organization (WHO) and food and agriculture organization of the United Nations (FAO) have stated that there is satisfactory scientific evidence to indicate that specific strains are safe and have the potential to provide the health benefits (**FAO/WHO, 2001)**. The aim of this work is the evaluation of the antimicrobial activity of lactobacilli isolated from fresh cow's milk against *S. aureus* which is a major mastitis pathogen

MATERIAL AND METHODS

1. Materials:

Milk samples collection according to **Oliver *et al.*, (2004).** The 105 tanks milk samples were collected under complete aseptic conditions into sterile bottles and then transported to the laboratory for investigation within 2 hours from dairy farms applying machine milking.

II. Method:

1. Isolation of lactic acid bacteria:

On MRS broth and agar media (Hi-Media, India), incubated anaerobically in an Anaerobic Gas-Pack system (Oxoid) for 48 hours at 37^{o}C to obtain single discrete colonies (**Halder *et al.*, 2015)**

2.Identification of bacterial isolates:

2.1. Phenotypic identification:

The purified bacterial colonies were identified by checking their macroscopic and microscopic appearance, biochemical reactions (catalase and oxidase) and motility test as described in Bergey's manual of systematic bacteriology (**Logan and De Vos, 2009**).

2.2. Molecular identification

Amplification of 16S rRNA gene was performed by a modified method of **Massol-Deya *et al.*, (1995).**

2.2.1. DNA extraction

A loopful of overnight grown cells was transferred to 50µl TE buffer and boiled for 5min, then centrifuged at 10,000 rpm for 10 min at 4°C. After that, 1µl of supernatant was used as template for PCR reaction.

2.2.2. Amplification of 16S rDNA Region by Polymerase Chain Reaction (PCR)

PCR was performed by using the Premix *Taq* (Ex *Taq* Version, Takara, Japan) according to instruction manual. A pair of flanking sequences was used for primer binding sites to partially amplify target 16S rRNA gene from the bacterial isolates, primers are LbLMA1-rev (5'-CTC AAA ACT AAA CAAAGT TTC-3') and R16-1 (5'-CTT GTA CAC ACC GCC CGT CA-3') (**Dubernet *et al.*, 2002**), *Lactobacillus acidophilus* La-5 was the reference strain used in this PCR methode . DNA fragments were amplified as follows: Amplification reactions were executed in total volume of 25µl containing 1µl of each primer (10pmol), 12.5µl of Premix *Taq* and 1µl of DNA template. PCR was carried out in genius model FGENO2TD thermal cycler (Techne, England).

The PCR conditions were accustomed to initial denaturation for 5min at 95°C then 30 cycles designed as (Denaturation:30 sec at 95°C, annealing: 30 sec at 55°C, and extension: 30 sec at 72°C), and finally 7 min at 72°C. The amplified products were subjected to electrophoresis in 1% agarose gels (Elec trophoresis grade, Invitrogen) in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2). Gels were stained with ethidium bromide (5 Wg ml31) and visualised by Gel Documentation system. (InGenius 3).

2.3. Identification using MALDI-TOF MS:

Pure cultures were identified to the species level using MALDI-TOF MS method. The samples were automatically analyzed using a MALDI-TOF mass spectrometry (Bruker, Germany) running Flexcontrol 3.4 software. The mass spectra of the tested samples were adjusted using the Bruker's bacterial test

standard (Bruker Daltonics) as described by **Nacef *et al*., (2016).**

3. Evaluation of probiotic activity:

3.1. Preparation of cell free supernatant (CFS):

Lactobacilli strains were cultivated in MRS broth for 48 h at 37°C. CFS was obtained by centrifuging the culture (10000 rpm, 10 min, 4°C) followed by filtration of the supernatant through a 0.2 µm pore size filter (**Nowroozi and Mirzaii, 2004**).

3.2. Well diffusion assay:

An overnight culture of bovine mastitis *S. aureus* (kindly provided by DR. Ebtsam Kotb, ARRI) was swabbed on the surface of nutrient agar plates (1.5 x 10^8 CFU/ml) where wells (of 6 mm diameter) were cut off, bottom sealed with 2 drops of soft agar and CFS (100 µL/well) of the isolated lactobacilli were loaded in the wells marked properly with the isolates' names, plates were left to dry then incubated for 24 hours at 37°C. After incubation the inhibition zone was recorded, interpreted as weak inhibition (≤10) mm, moderate inhibition (11-14) mm and strong inhibition (≥15) mm. The tests were performed in triplicates and the data were represented with mean ± SD, (**Mami *et al*., 2008 and Halder *et al.*, 2017)**.

3.3. Modified double layer method according to Soleimani *et al.*, (2010):

An overnight culture of each probiotic *Lactobacillus* in MRS broth at 37°C was prepared. 100 µl of each probiotic *Lactobacillus* culture (1.5 x 10^8 CFU/ml) was spotted onto the surface of MRS agar and incubated for 48 h at 37°C anaerobically. The plates of MRS agar containing lactobacilli spots were overlaid with 15 ml melted Muller Hinton agar and allowed to solidify. 100 µl of BM *S. aureus* individually streaked by swab over the entire agar surface. The plates were incubated for 24 h at 37°C. After incubation, the zone diameter of inhibition (ZDI) values were measured and interpreted as recorded by **Shokryazdan *et al*., (2014)** where the ZDI <10 mm, 10–20 mm and >20 mm were considered as weak, moderate and strong inhibitions, respectively.

4. Evaluation of safety profile of isolated lactobacilli:

Only Isolates with antibacterial activity against *S. aureus* were checked for their safety profiles using the following techniques:

4.1. Hemolytic Activity :

With some modification, MRS agar was used as a blood base for hemolytic activity assessment. The plates were observed for the formation of any clear zones (β-hemolysis), greenish hemolytic zones (α-hemolysis), or no such zones (γ-hemolysis) around the *Lactobacillus* colonies **(Halder *et al*., 2017)**.

4.2. Gelatin hydrolysis test:

To detect the ability of an organism to produce gelatinase (proteolytic enzyme) that liquefy gelatin **(Tille and Forbes, 2014)**.

4.3. Antibiotic susceptibility test:

The antibiotic susceptibility test was performed following disc diffusion method as described by **Bauer *et al*., (1966)**. The isolated strains were tested for their resistance against 8 antibiotics. The studied antibiotics were penicillin (P 30µg), tetracycline (TE 5µg), vancomycin (VA 30µg), gentamycin (CN 10 µg), ciprofloxacin (CIP 5µg), neomycin (N 30µg), amoxicillin/ clavulinic acid (AMC 30µg) and levofloxacin (LEV 10µg). The zone diameter of inhibition (ZDI) obtained around the antibiotic disc were recorded, and isolates were regarded as sensitive (ZDI; ≥21 mm), moderate (ZDI; 16-20 mm), or resistant (ZDI; ≤15 mm), (**Vlkova*, et al.*, 2006 and Liasi*, et al.*, 2009).**

RESULTS

1. Phenotypic identification:

One hundred- forty-six (146) isolates were identified as lactic acid bacteria (LAB) from 105 milk tanks samples, table 1. The isolated LAB appeared macroscopically as small to medium size smooth colonies white or creamy in color, Fig. 1 and microscopically they were Gram positive, non-spore forming rods or cocci varying in length and thickness, single, paired or may be in chains, Fig. 2. All were catalase, oxidase negative and non-motile.

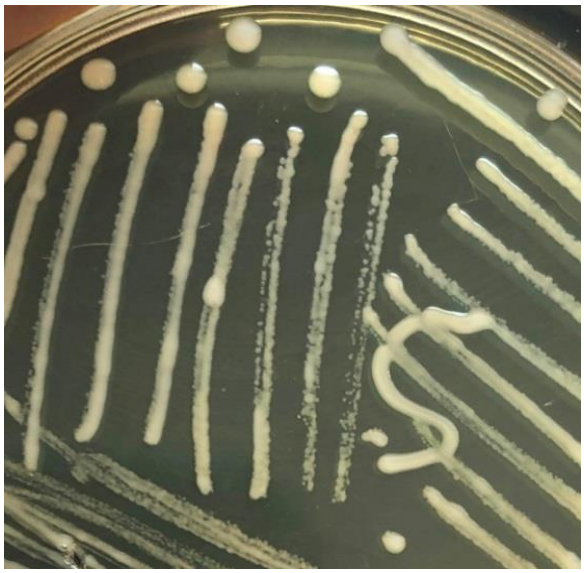

Fig. 1: LAB on MRS agar plates

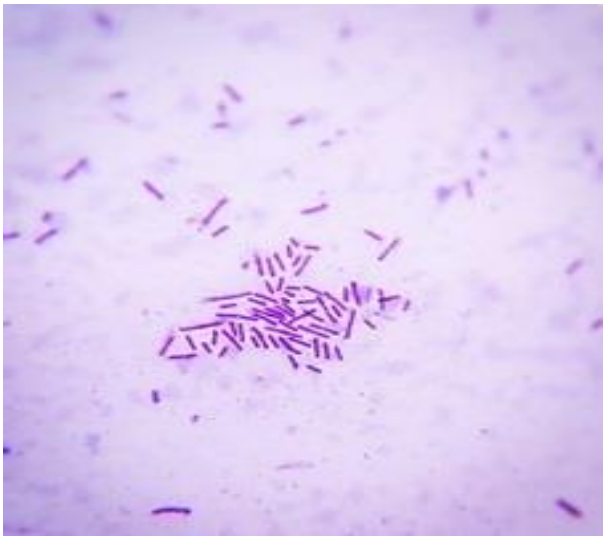

Fig. 2: Gram positive rods

Table 1: Bacteriological isolates from milk samples

No. of milk samples	LAB (lactic acid bacteria)		Total
	Rods	Cocci	
105	27	119	146

2. Molecular identification:

Rod-shaped isolates were introduced to (PCR) and were identified to the genus level using PCR method revealing that 24 isolates belong to genus *Lactobacillus*. PCR products presented a 250 bp amplicon approximately as show in Fig. 3 and table 2.

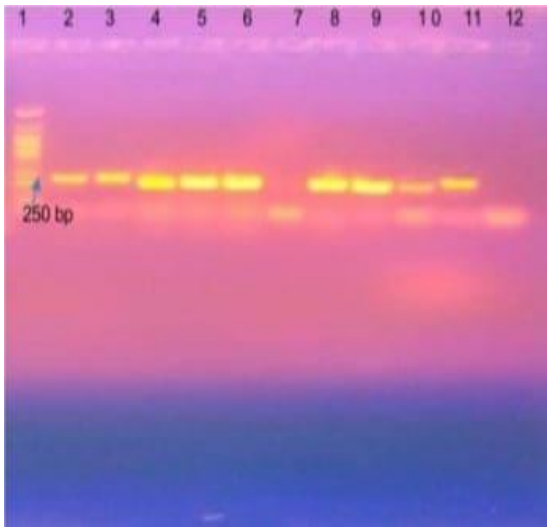

Fig. 3: PCR ampilification products, lane 1: 100 bp ladder, lanes from (3-6) and from (8-11) lactobacilli, lane 2: positive control (*Lactobacillus acidophilus* La-5), lane 12: negative control, lane 7: negative result.

3. Identification using MALDI-TOF MS:

The isolates were successfully identified by Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry to species levels as shown in table. 2 .

Table 2: Results of PCR and MALDI-TOF identification

Isolate serial	**PCR results**	**MALDI-TOF MS results**
B, O, G, S, W	*Lactobacillus* species	*L. fermentum*
A, U, N	*Lactobacillus* species	*L. brevis*
Y, V, M, C	*Lactobacillus* species	*L. plantarum*
D, I	*Lactobacillus* species	*L. paracasei*
R, F, T	*Lactobacillus* species	*L. rhamnosus*
P, X	*Lactobacillus* species	*L. pentosus*
Q, Z, J	*Lactobacillus* species	*L. casei*
K	*Lactobacillus* species	*L.raffinolactis*
L	*Lactobacillus* species	*L.mesenteroides*

4. Evaluation of antimicrobial activity of probiotic:

4.1. Agar well diffusion assay:

L. casei, *L. plantarum* and *L. fermentum* showed the highest inhibition while *L. brevis*, *L. mesenteriods*, *L. raffinolactis* and *L. paracasei* showed weak or no inhibition against *S. aureus*, Table 3 and Fig. 4.

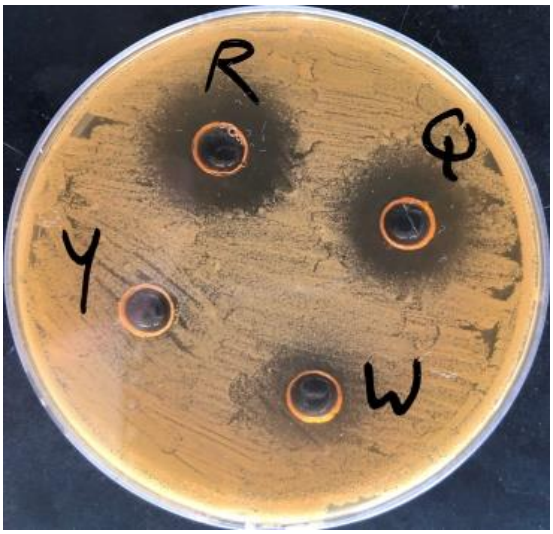

Fig. 4: Clear zones around the wells loaded with *Lactobacillus* cell free supernatant representing the inhibiting activity of *lactobacillus* against *S. aureus.* R= *L. rhamnosus*, Q=*L. casei*, W=*L. fermentum*, Y= *L. plantarum.*

Table 3: Agar well diffusion assay, results represent the mean ± standard deviation of three replicates.

Isolate serial	Isolate	*S. aureus* inhibition zone (mm)	Isolate serial	Isolate	*S. aureus* inhibition zone (mm)
P	*L. pentosus*	(- ve)	Z	*L. casei*	(-)
X	*L. pentosus*	++	Q	*L. casei*	+++
V	*L. plantarum*	+++	J	*L. casei*	(-)
Y	*L. plantarum*	(-)	L	*L. mesenteroide*	(-)
M	*L. plantarum*	+++	S	*L. fermentum*	(-)
C	*L. plantarum*	(-)	W	*L. fermentum*	+++
R	*L. rhamnosus*	+++	O	*L. fermentum*	+++
F	*L. rhamnosus*	(-)	G	*L. fermentum*	++
T	*L. rhamnosus*	++	B	*L. fermentum*	(-)
A	*L. brevis*	+	K	*L. raffinolactis*	(-)
U	*L. brevis*	+	I	*L. paracasei*	+
N	*L. brevis*	(-)	D	*L. paracasei*	(-)

Degree of inhibition: Week (+), moderate (++), strong (+++), no activity (-)

Table 4: Modified double layer method, antimicrobial activity of *Lactobacillus* against *S. aureus.*

Isolate serial	Isolate	*S. aureus* inhibition zone (mm)	Isolate serial	Isolate	*S. aureus* inhibition zone (mm)
P	*L. pentosus*	(-)	Q	*L. casei*	+++
X	*L. pentosus*	++	Z	*L. casei*	(-)
V	*L. plantarum*	+++	J	*L. casei*	(-)
M	*L. plantarum*	+++	L	*L. mesenteriods*	(-)
Y	*L. plantarum*	+	S	*L. fermentum*	(-)
C	*L. plantarum*	(-)	W	*L. fermentum*	+++
R	*L. rhamnosus*	+++	O	*L. fermentum*	+++
T	*L. rhamnosus*	++	G	*L. fermentum*	++
F	*L. rhamnosus*	(-)	B	*L. fermentum*	(-)
A	*L. brevis*	+	K	*L. raffinolactis*	(-)
U	*L. brevis*	+	I	*L. paracasei*	+
N	*L. brevis*	(-)	D	*L. paracasei*	+

Degree of inhibition: Week (+), moderate (++), strong (+++), no activity (-)

4.2.Modified double layer method:

L. casei, *L. rhamnosus*, *L. plantarum* and *L. fermentum* have the highest inhibitory effect on *S. aureus*, while *L. brevis*, *L. mesenteriods*, *L. raffinolactis* and *L. paracasei* showed weak or no inhibiting, shown in the table 4 and Fig. 5.

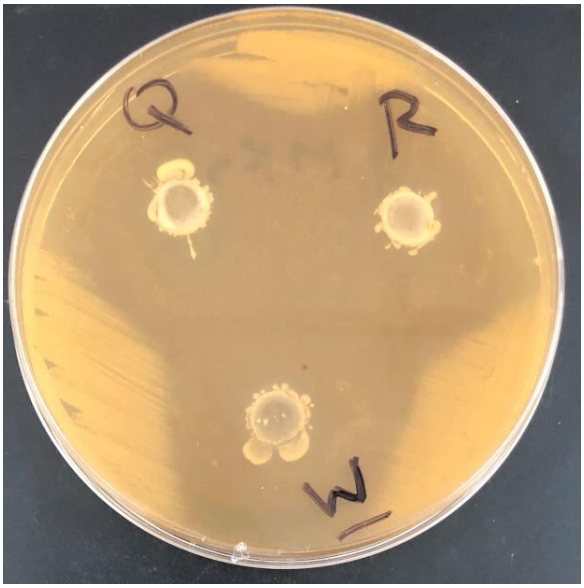

Fig. 5: Modified double layer method. Clear zones around the wells containing Lactobacillus growth representing the inhibiting activity of *lactobacillus* against *S. aureus.* R= *L. rhamnosus*, Q=*L. casei*, W=*L. fermentum.*

5. Evaluation of safety profile of isolated *lactobacilli*:

Isolates that showed inhibitory activity against the test subject (*S. aureus*) were checked for their safety by detecting their hemolytic activity, gelatin hydrolysis and antibiotic susceptibility.

5.1. Hemolytic Activity:

The isolated strains that showed inhibitory activity found to be non-hemolytic, as they didn't show any clear or greenish zones around the colonies that were grown on MRS blood agar plates (Fig. 6).

Fig. 6: Lactobacilli colonies on MRS blood agar plates showing γ –hemolysis.

5.2.Gelatin hydrolysis:

All of the isolated strains that showed inhibitory activity against *S. aureus* lack the ability to liquefy gelatin

5.3. Antibiotic susceptibility test:

The result of the antibiotic susceptibility test revealed that all the *Lactobacillus* isolates showed resistance to vancomycin (VA), neomycin (N) and gentamycin (CN), on the other hand, they were sensitive to amoxicillin clavulanic acid (AMC), Levofloxacin (LEV), tetracycline (TE) and penicillin (P) as shown in the Table 5, and Fig. 7.

Table 5: Antibiotic susceptibility test results

Isolate serial / antibiotic / isolate		Diameter of Zone of inhibition in millimeter(mm)							
		Amc (30µg)	CIP (5µg)	CN (10µg)	LEV (10µg)	N (30µg)	TE (5µg)	VA (30µg)	P (30µg)
X	*L. pentosus*	S	R	R	R	R	R	R	S
M	*L. plantarum*	S	R	R	R	R	R	R	S
V	*L. plantarum*	S	R	R	R	R	R	R	S
R	*L. rhamnosus*	S	M	R	S	R	S	R	S
T	*L. rhamnosus*	S	M	R	S	R	S	R	S
Q	*L. casei*	S	M	R	S	R	S	R	S
W	*L. fermentum*	S	R	R	M	R	S	R	S
O	*L. fermentum*	S	R	R	M	R	S	R	S

Amc= amoxicillin clavulanic acid, CIP= ciprofloxacin, CN= gentamicin, LEV= Levofloxacin, N= neomycin, TE= tetracycline, VA= vancomycin, P= penicillin, Sensitive= S, moderate=M, resistant=R.

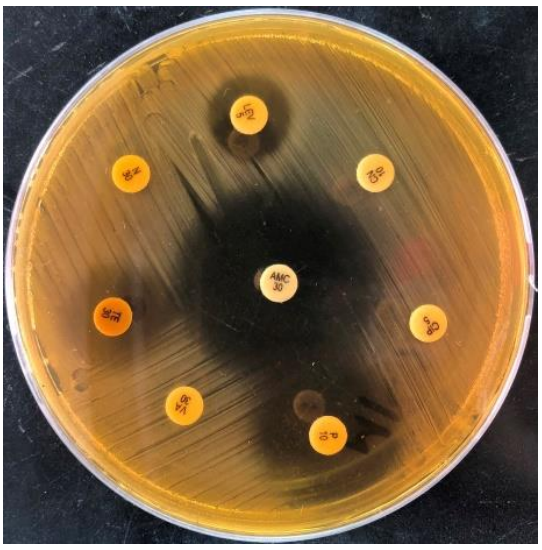

Fig. 7: Antibiotic susceptibility test for *L. plantarum*, Inhibition zones around antibiotic disc

DISCUSSION

It has become a popular theory to use probiotics for their beneficial effect in recent years, where researchers are studying how lactobacilli interact and affect their host. **(Reardon, 2014).** Moreover; the isolation and screening of lactobacilli from numerous natural sources is a successful way to develop new probiotic strains with valuable medicinal significance (**Halder *et al.*, 2017).** So, this study highlights the detection of antibacterial activity of *Lactobacillus* spp. isolated and identified from fresh cow's milk against *S. aureus* which is a major mastitis pathogen, and studied their safety profiles. A total of 146 isolates were identified phenotypically and biochemically as lactic acid bacteria (LAB) from 105 tanks milk as shown in table (1) after being cultured on MRS agar plates and incubated for 48 hours anaerobically depending on the phenotypic and biochemical features.

Our results were similar to those recorded by **Touret *et al.*, (2018); Mostafa *et al., (*2019); Vanniyasingam *et al*., (2019) and Jobby *et al., (*2020)** whom were able to isolate strains that match the phenotypic and biochemical features of LAB. Relying on conventional methods based on phenotypic, physiological and biochemical criteria to identify LAB is complex and time-consuming approaches and can underestimate the microbiological diversity of a bacterial community **(Ercolini *et al*., 2001).**

In contrast, advanced methods as the nucleic acid-based molecular methods have been recognized to be prevailing tools for the identification of bacterial isolates, and that motivated researchers to give attention to molecular biology applications for the differentiation and rapid detection of LAB **(Coeuret *et al.*, 2003).** So, to confirm the genus identification of the isolates we applied PCR technique which helped to identify the lactobacilli at the genus levels as 24 LAB isolates were successfully identified to belong to genus *Lactobacillus* with the aid of genus-specific primers LbLMA1-rev and R16-1. PCR products presented a 250 bp amplicon approximately as show in table (2) and figure (3). These results were similar to those obtained by **Dubernet *et al.*, (2002); Sulieman *et al., (*2007)** and **Touret *et al.*, (2018).**

As for further identification of the isolated *Lactobacillus* to species level, MALDI-TOF MS technique was applied. Using MALDI-TOF MS is ought to be suitable for the identification of anaerobic bacteria **(Veloo *et al.*, 2011).** In the current study, the precise identification of the 24 isolates using MALDI-TO MS is (3) *L. casei* strains, (4) *L. plantarum* strains , (5) *L. fermentum* strains, (3) *L. brevis* strains,(1) *L. mesenteriods* strains, (1) *L. raffinolactis* strains, (2) *L. paracasei* strains,(2) *L. pentosus* strains and (3) *L. rhamnosus* strains as shown in table (2).

This technique is considered a fast, cost-effective and dependable method **(Pavlovic *et al*., 2013).** It is, therefore, considered to be an excellent substitute to biochemical and even molecular methods (**Dec *et al*., 2014; Vithanage *et al*., 2014**). **Dušková *et al.*, (2012)** stated that the MALDI-TOF MS technique has an advanced success rate (93%) than the polymerase chain reaction (PCR) method (77%) in recognizing *Lactobacillus* at the species level. Moreover, this approach is becoming a method of choice for defining the genus, species and even subspecies levels of bacterial isolates **(Carbonnelle *et al.*, 2012).** Also, this technique can be applied for the identification of other microorganisms as yeasts and fungi isolated from different sources **(Chalupova *et al.*, 2014)**. Using MALDI-TOF MS, **(Nacef *et al.*, 2016); Kanak** and **Yilmaz, (2018) and Mostafa *et al.*, (2019)** were able to identify *L. rhamnosus, L. brevis, L. paracasei, L. plantarum L. fermentum, L. curvatus, L. fructivorans* and *L. parabuchneri* from different samples including raw milk.

Antimicrobial activity is one of the most significant selection criteria for LAB to be used as a probiotic (**Klaenhammer and Kullen, 1999)** through the production of inhibitory compounds that antagonize pathogenic bacteria **(Nemcova *et al*., 1997; Jacobsen *et al.*, 1999). Ryan *et al*., (1999)** stated that using non-antibiotic preparations to combat bovine mastitis can diminish the necessity of using antibiotics in treatment of such disease, so the problem of the development of antibiotic resistance pathogens can be solved to a great extent.

In this study, the antimicrobial activity of the isolated lactobacilli against one of the major causes of bovine mastitis, *S. aureus*, was investigated by using two methods, the first method is agar well diffusion assay. The results are shown in table 3 and Fig. 4 as *L. casei, L. plantarum, L. fermentum* and *L. rhamnosus* showed the highest inhibition. The findings of **Mostafa *et al.*, (2019)** and **Jobby *et al., (*2020)** confirmed these results. The second method was modified double layer method which is shown in table 4 and Fig. 5 where the best inhibition zones against *S. aureus* were achieved in the presence of *L. casei, L. fermentum, L. plantarum* and *L. rhamnosus.* These results were supported with the findings published by **Mami *et al.,(* 2008)** and **Soleimani *et al.*, (2010).**

According to **Tambekar *et al.*, (2009) and Halder *et al., (*2017),** this inhibitory effect occurred as a result of the ability of LAB, especially the lactobacilli to product some antimicrobial compounds as organic acids (lactic acid and acetic acid), diacetyl, hydrogen peroxide, bacteriocins and bacteriocin- like substances which were produced during the course of the experiment in addition to their competition for

nutrients .From the results obtained by the previous two methods to evaluate the antibacterial activity of the isolated lactobacilli, it was clear that the diameter of the inhibition zones in the modified double layer method is nearly twice the diameter of inhibition zones obtained from the agar well diffusion method, this is may be due to the production of inhibitory compounds which were induced more by the existence of the pathogen in contact with the *Lactobacillus* (as occurred in modified double layer method), in some cases, co-culture of LAB with target pathogen can be required for the production of bacteriocin (**Cotter *et al.*, 2005** and **Touret *et al.*, 2018).**

Evaluating the safety profile of a *Lactobacillus* strain is essential to determine whether if it can to be used as a probiotic or not, it must be non- pathogenic or have any harmful effects to the host, moreover, it should be GRAS and have a beneficial effect to the host **(Fuller, 1989).** The probiotic, like lactobacilli, must lack the ability to cause hemolysis as well as liquefaction of gelatin inside host body. The hemolysis and gelatin liquefaction remain on of the major virulence features amongst pathogenic bacteria **(Halder *et al.*, 2017).**

In the current study, the isolated strains that showed inhibitory activity against *S. aureus* found to be non-hemolytic (γ –hemolysis) as shown in figure (6), as they didn't show any clear or greenish zones around the colonies that were grown on MRS blood agar plates, also, they lack the ability to liquefy gelatin. Our results were confirmed by **Mami *et al.*, (2008)** and **Halder *et al.*, (2017).** On the other hand **Touret *et al.*, (2018)** isolated 59 lactobacilli from fermented Sauerkraut and found that only one was β- hemolytic, 18 strains showed α-hemolysis and 40 isolate presented γ-hemolysis. Antibiotic susceptibility profile of an isolate can be used to assess its safety to be used as a potential probiotic **(Georgieva *et al.*, 2015).**

In the present study, the results of the antibiotic susceptibility test showed that all *Lactobacillus* isolates were resistance to vancomycin (VA), neomycin (N) and gentamycin (CN), *L. plantarum and L. pentosus* expressed resistance against ciprofloxacin (CIP). On the other hand, all of the isolated strains were sensitive to amoxicillin clavulanic acid (AMC), Levofloxacin (LEV), tetracycline (TE) and penicillin (P). as shown in table (5), figure (7) and (8). **Halder *et al.*, (2017); Touret *et al.*, (2018)** and **Jobby *et al.*, (2020)** confirmed our results as all the strains that they isolated were found to be resistant to vancomycin, which is regarded as an intrinsic(non-transferable) or natural resistance. **Vanniyasingam *et al.*,(2019)** recommended that the variation in results may be due to different sources used for the isolation of LAB and vary from strain and another.

Halder *et al.*, (2015) recommended that lactobacilli strains possessing resistance to antibiotics might be suitable for co-administration along with antibiotics in preventing antibiotic-induced diarrhea and/or in the treatment of intestinal illnesses. Lately, some commercial antibiotics are being manufactured containing a combination of antibiotics in addition to probiotic bacteria as (DOXY-1 L-Dr FORTE by USV Ltd. and AVIDOX-LB by Avalanche pharmaceuticals) which contains Doxycycline and *Lactobacillus.*

CONCLUSION

Strains (M, V) *L. plantarum,* (R) *L. rhamnosus,* (Q) *L. casei* and (W, O) *L. fermentum* are good candidates to be used as probiotic regarding to their antibacterial activity against *S. aureus* and their safety profiles. Also, MALDI-TOF MS is considered an accurate, affordable and a rapid method for identification of members of genus *Lactobacillus.* The use of vancomycin for selective isolation of *Lactobacillus.* Lactobacilli strains possessing resistance to antibiotics might be suitable for co-administration along with antibiotics to help in controlling bovine mastitis.

Statement of conflict of interests

All authors declare there is no conflict of interest.

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