

Isolation, molecular characterization and antibiogram of Lactic acid bacteria from dairy cattle sources of Mizoram, India

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ABSTRACT

A total of 250 samples of dairy cattle sources comprised of 100 cattle faeces, 75 raw milk, and 75 fermented milk samples which were randomly collected from different areas of Aizawl district of Mizoram that have been analyzed bacteriologically for enumeration and isolation of Lactic acid bacteria (LAB). The 90 phenotypically positive LAB isolates were further analyzed molecularly by *16S-rRNA* gene analysis and 42 isolates were found positive. A total of 20 PCR positive LAB isolates were randomly selected and sequenced, out of which 11 isolates were positive for LAB after sequence analysis, belonging to six species of LAB, namely *Lactibacillus plantarum* (4), *Lactobacillus fermentum* (2), *Lactobacillus brevis* (2), *Bacillus coagulans* (1), *Enterococcus faecium* (1) and *Weissella cibaria* (1). The phylogenetic tree was constructed to check the relatedness of the strains with other referral LAB strains from NCBI gene bank. These 11 isolates were further analyzed for antibiogram. All 11 LAB strains tested for antibiotic sensitivity were 100% resistant to kanamycin, whereas intermediate resistance was shown by *Lactobacillus brevis* FM046 to clindamycin and three strains namely *Lactobacillus fermentum* FM011, *Bacillus coagulans* FM033 and *Lactobacillus brevis* FM046 to penicillin. All the 11 LAB strains were 100% sensitive to most of the tested antibiotics namely amikacin, ampicillin, azithromycin, cefoxitin, cefpodoxime, ceftriaxone, erythromycin, gentamicin and rifampicin. The LAB strains detected from the dairy cattle sources of Mizoram with sensitive antibiogram might be further studied for their probiotic potential.

Keywords: Dairy cattle, Lactic acid bacteria, Mizoram, Phylogenetic analysis, *16S-rRNA* gene sequencing

Lactic acid bacteria (LAB) are a group of gram-positive, non-spore forming, non-motile coccus or rod shaped, catalase-negative and acid-tolerant facultative anaerobes (Van *et al.* 1998). The LAB are microorganisms that mainly produce lactic acid as a by-product during metabolic activities (Bintsis 2018). Based on the products of the fermented carbohydrates, LAB are divided into homofermentative and heterofermentative bacteria. *Pediococcus*, *Lactococcus* and *Streptococcus* are homofermentative bacteria and lactic acid is the sole result of glucose fermentation, whereas *Leuconostoc* and *Weissella* are heterofermentative bacteria that produce carbon dioxide (CO₂), lactate and ethanol from glucose (Caplice and Fitzgerald 1999).

Soil and plants as well as the guts of herbivorous animals are thought to be the ancient LAB's original niche (Morelli *et al.* 2019). Resistance of these organisms to host barriers, attachment to intestinal cells and fermentation of certain substrates in the gut are three areas of genomic adaptation during the transfer from soil and plants to the animal gut (Lebeer *et al.* 2008).

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The LAB are members of the intestinal microbiota of vertebrates including humans and participate in the fermentation of a variety of foods, increasing food quality and safety as well as consumer health and comfort. These microorganisms are GRAS (generally recognized as safe) and can be used as probiotics (Halder *et al.* 2017). They play a multifaceted role in the agricultural, food and clinical sectors (Bintsis 2018). The bio-preservation of foods using bacteriocinogenic LAB isolated directly from foods is another innovative approach (Yang *et al.* 2012). LAB has a variety of health benefits for the host including better immunological function, digestion, management of inflammatory bowel illnesses, constipation relief and strengthening of the mucosal barrier (Li *et al.* 2020). They are capable of suppressing pathogenic organisms' growth through a variety of ways including adhesion to epithelial cells, immune system regulation and antimicrobial chemical release (Somashekaraiah *et al.* 2019).

Antibiotic abuse in domestic animal feeding has posed a serious threat to animal health and welfare as well as the human health and environment in recent decades. Probiotic LAB have become one of the most extensively used feed additives as an alternative to antibiotics since they help to avoid certain diseases and maintain good health (Li *et al.* 2020). The LAB isolated from native sources which are

sensitive to commonly used antibiotics may be explored as a source of potential probiotics.

The present study aims at enumeration, isolation and molecular characterization of LAB from dairy cattle sources of Mizoram, India.

MATERIALS AND METHODS

Collection of samples: A total of 250 samples of dairy cattle sources including 100 faeces, 75 raw milk and 75 fermented milk samples were randomly collected during the year 2022 by following aseptic measures from different areas of Aizawl district of Mizoram and brought to the laboratory of Department of Veterinary Public Health & Epidemiology, College of Veterinary Sciences & Animal Husbandry, Selesih, Aizawl in appropriate cold chain and immediately subjected for bacteriological analysis for enumeration and isolation of LAB.

Isolation of LAB: The enumeration of LAB and isolation of LAB pure culture were carried out in accordance with the method described by Khalil and Anwar (2016) with minor modifications. One ml/g of the sample was homogenized with 9 ml of De Man, Rogosa and Sharpe (MRS) broth to make an initial dilution of 10^{-1} . The samples were serially diluted up to 10^{-6} . A volume of 0.1 ml of the appropriate dilution was inoculated by spreading on MRS agar plate and was incubated at 37°C for 24 h for the enumeration of LAB. The plates that showed 30-300 colonies were selected for enumeration and the results were expressed as colony forming units (CFU) per ml/g by multiplying the average number of colonies with the reciprocal of dilution. The sample showing a count of 10^6 was considered for isolation of the pure culture of LAB. Such colonies showing Gram positive and catalase negative reactions were phenotypically identified as LAB and subjected to molecular characterization and antibiogram studies.

Identification of the LAB species by 16S-rRNA gene analysis: The DNA lysate was prepared from the pure culture of presumptively identified LAB strains by using a DNA purification kit [HiPurA Food Pathogen (Bacteria) DNA purification kit (HIMEDIA)] as per the manufacturer's protocol. The DNA lysates of the LAB isolates were subjected to 16S-rRNA gene analysis according to the method described by Yadav *et al.* (2016) by using published primers (F-AGAGTTTGATCATGGCTCAG and R-CGGTATTAGCATCTGTTTCC) of 200 bp.

In a thermal cycler machine (Bio-Rad) with a preheated lid, DNA was amplified by following the cycling conditions for Initial denaturation at 95°C for 5 min, denaturation 95°C for 30 s, annealing at 55°C for 30 s, extension 72°C for 30 s and final extension for one cycle to a total of 34 cycles. The amplified PCR products were kept at 4°C and subsequently examined by agar gel (1.5%) electrophoresis.

Sequence analysis of the LAB-PCR products: The PCR amplified LAB products were purified using a commercial PCR purification kit (GeneJET PCR Purification Kit, ThermoScientific) according to the manufacturer's protocol. Then the purified PCR products were sent for

commercial sequencing at Unipath Speciality Laboratory Ltd, Ahmedabad, India.

Phylogenetic analysis of the sequenced DNA from LAB isolates: The phylogenetic analyses of the sequenced DNA were done by using MEGA 11 software. The referral sequences taken from NCBI were aligned by the muscle alignment system in MEGA 11 software. These aligned sequences were then taken for construction of phylogenetic tree by neighbour-joining using the bootstrap method with a number of bootstrap replications of 1000.

Evaluation of the antibiotic susceptibility of isolated LAB isolates: The antibiotic susceptibility of the LAB isolates was tested using the disc diffusion method (Bauer *et al.* 1966). A panel of 12 antibiotics, namely amikacin (30 µg), ampicillin (10 µg), azithromycin (15 µg), cefoxitin (30 µg), cefpodoxime (10 µg), ceftriaxone (30 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), penicillin G (10 units) and rifampicin (5 µg) were used in the present study.

A single colony of LAB isolate was picked up and dispensed into a 0.85% (w/v) sterile sodium chloride (NaCl) solution. The bacterial suspension was uniformly distributed on Mueller Hinton agar (MHA) plates with a thickness of 4 ± 0.5 mm by using a sterile cotton swab. Standard antibiotic discs (6 mm in diameter) were placed on the medium and plates were incubated at 37°C for 48 h. Results were observed by measuring the zone of inhibition.

Statistical analysis: The data obtained were analyzed using statistical package SPSS version 27.0.

RESULTS AND DISCUSSION

Enumeration, isolation and molecular detection of LAB from dairy cattle faeces, raw milk and fermented milk: Out of the 250 samples including 100 dairy cattle faeces, 75 raw milk samples and 75 fermented milk samples, 109 (43.60%) samples were culturally positive, including 39 dairy cattle faeces, 24 raw milk and 46 fermented milk. On phenotypic analysis of 109 isolates, 90 (36.00%) isolates were Gram positive and catalase-negative and presumptively identified as LAB. Phenotypically positive LAB isolates exhibited desired cultural characteristics such as change in colour of MRS broth from clear yellow colour broth to turbid yellow and growth of tiny opaque creamy distinct colonies on MRS agar. Morphologically, the colonies were risen from the centre and were white with smooth edges. On molecular analysis of the 90 presumptive LAB isolates by 16S-rRNA gene (Fig.1) by PCR, 42 (16.80%) isolates showed specific bands (200 bp) (Table 1). These 42 PCR positive LAB isolates showed the colony counts in the range between 6.19 ± 0.09 and 8.25 ± 0.02 log cfu/ml. The viable colony counts of the LAB isolates more than 10^6 included 11 cattle faeces isolates ranging between 6.31 ± 0.03 and 7.28 ± 0.04 log cfu/ml, 12 raw milk isolates ranging between 6.26 ± 0.05 and 7.41 ± 0.05 log cfu/ml and 19 fermented milk isolates ranging between 6.19 ± 0.09 and 8.25 ± 0.02 log cfu/ml.

There was a significant difference ($p \leq 0.05$) in phenotypic

Table 1. Detection of LAB by phenotypic and molecular methods from dairy cattle, raw milk and fermented milk in Mizoram

Types of samples	No. of samples analyzed	Phenotypic method		Molecular method		Percentage (%)	
		Culturally positive		Biochemically positive	No. of samples positive for <i>16S rRNA</i> gene		
		No. of samples showing growth >10 ⁶ cfu/gm or cfu/ml	No. of samples showing Gram's positive reaction	No. of samples showing Catalase test negative			
Dairy cattle faeces	100	39	39	27	27	11	13.00
Cattle milk	75	24	24	21	21	12	16.00
Fermented milk	75	46	46	42	42	19	22.66
Total	250	109	109	90	90	42	16.80

Cultural: $\chi^2=0.01^*$ $\chi^2_{12}=0.34$ $\chi^2_{13}=0.00^{**}$ $\chi^2_{23}=0.00^*$ Biochemical: $\chi^2=0.02^*$ $\chi^2_{12}=0.09$ $\chi^2_{13}=0.00^{**}$ $\chi^2_{23}=0.61$ molecular: $\chi^2=0.51$.

detection rate of LAB isolated from different sources but not among PCR positive isolates and the highest number of LAB isolates were obtained from fermented milk (22.66%) followed by raw milk (16.00%) and cattle faeces (13.00%) (Table 1).

Earlier to the present study, Adetoye *et al.* (2018), Bin *et al.* (2018) Maldonado *et al.* (2018), Guo *et al.* (2020), Lin *et al.* (2020), Pawar *et al.* (2020) and Taye *et al.* (2021) isolated the presumptive LAB based on the colony morphological studies and biochemical characteristic like Gram positive and catalase negative reaction. Adetoye *et al.* (2018) found a total of 88 LAB belonging 4 genera and 15 species were isolated and identified from cattle faeces. Taye *et al.* (2021) obtained a total of 41 bacterial isolates categorized under five different genera of LAB and identified from raw milk, cheese and yoghurt. Similarly, Yadav *et al.* (2016) from India isolated 54 isolates on MRS agar from the milk product, raabadi.

Molecular characterization of the LAB isolates from dairy cattle faeces, raw milk and fermented milk: Out of the 20 randomly selected PCR positive LAB isolates (7 from dairy cattle faeces, 6 from raw milk and 7 from fermented milk) subjected to *16S-rRNA* gene sequencing, 11 (55.00%) isolates were sequence positive for the LAB including 3 (42.85%) from dairy cattle faeces, 3 (50.00%) from raw milk and 5 (71.42%) from fermented milk. Upon NCBI BLAST analysis of these 11 sequences of *16S-rRNA* gene, six (6) species of LAB were detected, namely *Lactobacillus plantarum* (4), *Lactobacillus fermentum* (2), *Lactobacillus brevis* (2), *Bacillus coagulans* (1), *Enterococcus faecium* (1) and *Weissella cibaria* (1) (Table 2).

Phylogenetic trees of these 11 LAB strains were constructed by using neighbour joining method of *16S-rRNA* gene sequences (Fig. 2). Per cent similarities of the LAB strains with NCBI referral organism sequences are given in Table 3.

The present findings were found in accordance with Bin *et al.* (2018) who reported that *16S-rRNA* sequences

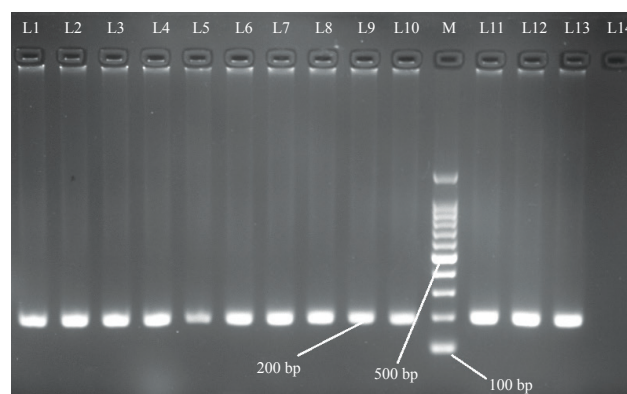


Fig. 1. Agarose gel electrophoresis showing PCR amplicons of *16S-rRNA* gene (200 bp); M: 100bp ladder; L14: Negative control; L13: Positive control; L1 to L12: Positive samples.

of 14 distinct species belonged to five genera, namely *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Streptococcus* and *Weissella* which were matched with the nucleotide sequences of 46 strains. Li *et al.* (2020) obtained 192 pure bacterial colonies from various gastrointestinal compartments of wild boars belonging to five species including *Lactobacillus mucosae*, *Lactobacillus salivarius*, *Enterococcus hirae*, *E. durans* and *E. faecium*. Bazireh

Table 2. The species of LAB detected in sequence analysis from different samples of dairy cattle sources in Mizoram

Strain number	Name of the strain detected	Sample source
CF016	<i>Enterococcus faecium</i>	Cattle faeces
CF022	<i>Lactiplantibacillus plantarum</i>	Cattle faeces
CF082	<i>Lactiplantibacillus plantarum</i>	Cattle faeces
CM022	<i>Lactiplantibacillus plantarum</i>	Cattle milk
CM056	<i>Weissella cibaria</i>	Cattle milk
CM072	<i>Lactiplantibacillus plantarum</i>	Cattle milk
FM011	<i>Lactobacillus fermentum</i>	Fermented milk
FM022	<i>Lactobacillus fermentum</i>	Fermented milk
FM033	<i>Bacillus coagulans</i>	Fermented milk
FM046	<i>Lactobacillus brevis</i>	Fermented milk
FM077	<i>Lactobacillus brevis</i>	Fermented milk

Table 3. Per cent similarity of the LAB stains with NCBI referral strains

LAB strains	NCBI referral accession no.	% Identity
<i>Enterococcus faecium</i> CF016	KF135665	100.00
	KC478509	100.00
<i>Lactiplantibacillus plantarum</i> CF022	CP053571	98.12
	AP019815	97.01
<i>Lactiplantibacillus plantarum</i> CF082	CP053571	98.51
	AP019815	98.51
<i>Lactiplantibacillus plantarum</i> CM022	CP053571	100.00
	AP019815	100.00
<i>Weissella cibaria</i> CM056	FN330974	100.00
<i>Lactiplantibacillus plantarum</i> CM072	CP053571	98.67
	AP019815	97.96
<i>Lactobacillus fermentum</i> FM01	KR816161	99.38
	OL354445	99.39
<i>Lactobacillus fermentum</i> FM022	KR816161	100.00
	OL354445	100.00
<i>Bacillus coagulans</i> FM033	MG55779	100.00
	HM35284	100.00
	AB362709	100.00
<i>Lactobacillus brevis</i> FM046	OP743923	98.00
	KU851157	99.25
	AB362611	99.25
<i>Lactobacillus brevis</i> FM077	OP743923	100.00
	KU851157	95.36
	AB362611	95.36

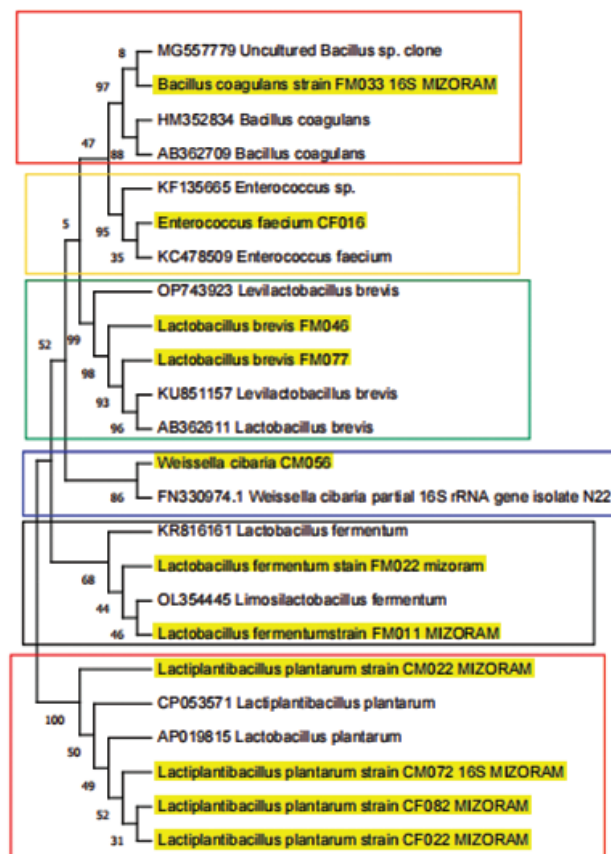


Fig. 2. Phylogenetic tree of LAB strains; highlighted strains were isolated in the present study.

et al. (2020) detected LAB by the PCR targeting the 16S-rRNA gene in human faeces.

Antibiotic sensitivity of the isolated LAB strains: All the 11 LAB strains were 100% sensitive to amikacin, ampicillin, azithromycin, cefoxitin, cefpodoxime, ceftriaxone, erythromycin, gentamicin and rifampicin whereas 100% resistant to kanamycin. Intermediate resistance was shown by *Lactobacillus brevis* FM046 to clindamycin and another three strains namely *Lactobacillus fermentum* FM011, *Bacillus coagulans* FM033 and *Lactobacillus brevis* FM046 to penicillin (Table 4).

Being a common component of Indian diet, fermented milk may bring massive volumes of live beneficial bacteria of LAB group into the human gut. Despite the fact that LAB have a long history of being widely employed in the creation of fermented milk and other fermented food products and are generally considered safe, some of them have been found to have innate or acquired antimicrobial resistance. As a result, it is vital to assess the antimicrobial resistance of LAB in various fermented foods (Clementi and Aquilanti 2011, Pan *et al.* 2011).

The isolated LAB strains were found to be resistant to kanamycin and sensitive to most of the other antibiotics studied. Nawaz *et al.* (2011) and Dec *et al.* (2020) had also observed the prevalence of kanamycin-resistant LAB strains of different species. It is well known that some *Lactobacillus* species exhibit resistance to aminoglycoside

due to the presence of aminoglycoside resistance gene, *aph(3'')-IIIa*, coding for the kinase *APH(3'')-IIIa* conferring resistance to kanamycin (Rojo *et al.* 2006). Nath *et al.* (2020) observed that *Lactobacillus plantarum* strain GCC-19M1 was susceptible to gentamicin, vancomycin, polymyxin-B, ofloxacin, ampicillin, norfloxacin, rifampicin, amikacin, penicillin-G, streptomycin, ciprofloxacin, azithromycin and ceftriaxone although the bacteria were found to be resistant to clindamycin. In contrast to the present findings, Stefanska *et al.* (2021) found that 19 LAB strains had phenotypic resistance to at least one antibiotic, while 15 bacteria had resistance against multiple drugs. The resistance was most commonly observed against aminoglycosides and tetracycline in 37% and 26% of the bacterial strains, respectively. The intermediate resistance of LAB strains towards penicillin and clindamycin might be due to the presence of the *bla* gene linked to resistance to β -lactam antibiotics and also the presence of *erm(B)* gene in LAB strains, respectively (Dec *et al.* 2018, Erginkaya *et al.* 2018).

The prevalence of LAB in dairy cattle, raw milk and fermented milk detected from Mizoram showed identification of six species of LAB with appreciable antimicrobial sensitivity indicating the future perspective of possible identification of probiotic LAB from dairy cattle sources for sustainable production of local fermented food products.

Table 4. Antibiotic sensitivity pattern of the LAB strains isolated from dairy cattle faeces, raw milk and fermented milk in Mizoram

Antibiotic	Sensitivity of the LAB strains										
	<i>Enterococcus faecium</i> CF016	<i>Lactiplantibacillus plantarum</i> CF022	<i>Lactiplantibacillus plantarum</i> CF082	<i>Lactiplantibacillus plantarum</i> CM022	<i>Weissella cibaria</i> CM056	<i>Lactiplantibacillus fermentum</i> CM072	<i>Lactobacillus fermentum</i> FM011	<i>Lactobacillus fermentum</i> FM022	<i>Bacillus coagulans</i> FM033	<i>Lactobacillus brevis</i> FM046	<i>Lactobacillus brevis</i> FM077
Amikacin (30 ug)	S	S	S	S	S	S	S	S	S	S	S
Ampicillin (10 ug)	S	S	S	S	S	S	S	S	S	S	S
Azithromycin (15 ug)	S	S	S	S	S	S	S	S	S	S	S
Cefoxitin (30 ug)	S	S	S	S	S	S	S	S	S	S	S
Cefpodoxime (10 ug)	S	S	S	S	S	S	S	S	S	S	S
Ceftriaxone (30 ug)	S	S	S	S	S	S	S	S	S	S	S
Clindamycin (2 ug)	S	S	S	S	S	S	S	S	S	I	S
Erythromycin (15 ug)	S	S	S	S	S	S	S	S	S	S	S
Gentamicin (10 ug)	S	S	S	S	S	S	S	S	S	S	S
Kanamycin (30 ug)	R	R	R	R	R	R	R	R	R	R	R
Penicillin G (10 units)	S	S	S	S	S	I	I	S	I	I	S
Rifampicin (5 ug)	S	S	S	S	S	S	S	S	S	S	S

S, No. of sensitive isolates; I, No. of intermediate isolates and R, No. of resistant isolates.

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