



Characterization of Lactic Acid Bacteria Isolated from *Idli* Batter and their Susceptibility to Antibiotics

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ABSTRACT

Idli is a common flour based fermented food prepared using lactic acid bacteria which bear probiotic properties, consumed in Sri Lanka and some parts of India. The objective of the present study was to characterize the lactic acid bacteria from *idli* batter, identify the changes of acidity during fermentation and to determine the antibiotic sensitivity of isolates. Lactic acid bacteria were isolated and characterized up to genus level. Subsequently, antibiotic susceptibility of isolates against four antibiotics was studied. Changes of pH, lactic acid bacterial count and titratable acidity with fermentation were determined. The pH value dropped steadily from 6.28 to 3.72 while titratable acidity increased from 0.24 to 0.92% during the fermentation period of 0 to 32 h. With fermentation, the lactic acid bacterial count was increased up to 9.88 log₁₀cfu/g after 12 hours of fermentation and the count reduced with increasing acidity. A bacterial count of 5.91 log₁₀cfu/g was recorded at the end of 32 hours of fermentation. Ten lactic acid bacteria isolates were identified from *idli* batter, of which six isolates were rod shaped and other four were cocci. All isolates were Gram positive, non-motile, non-spore formers and catalase negative. According to the above tests, it was confirmed up to the genus level that all strains belonged to Lactic acid bacteria. All isolates were susceptible to more than two antibiotics. The diameter of the zone of inhibition ranged from 1-23 mm. The highest zone of inhibition was observed in isolates I-6 against chloromphenicol (23 mm) while the lowest zone of inhibition was observed in isolates I-2 against erythromycin (1 mm). With fermentation the biochemical and sensory qualities of *idli* batter were changed. Lactic Acid Bacteria isolates showed different sensitivity towards antibiotics tested.

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INTRODUCTION

Fermentation is considered the oldest food preservation method yet fermented foods are popular even today across the world. In fact, fermented foods are gaining an ever-increasing momentum due to their health benefits. In fermentation, lactic acid bacteria (LAB) play a major role and the optimum amount of acid production varies with the product type.

Isolation and screening of new probiotic strains of LAB from fermented products is important as these micro flora may possess strong probiotic functionalities such as the ability to produce organic acids, exopolysaccharides, proteolytic products and production of antimicrobial activity against many food borne pathogens (El-Soda *et al.*, 2003; Ayad *et al.*, 2004; Abd El Gawad *et al.*, 2010 and Ayad and Shokery, 2011).

Even though the LAB are widely used and generally recognized as safe (GRAS) for usage and consumption, studies have shown that some of the LAB acquire antibiotic resistance (Clementi and Aquilanti, 2011). Further, the resistance character can be transmitted through the food chain in human and subsequently it may transfer to pathogenic bacteria. Hence, it is important to evaluate the antibiotic resistance of LAB before use them as food or starter cultures (Rodríguez-Alonso *et al.*, 2009).

Most traditional Asian fermented foods are non-dairy products prepared using different non-dairy raw materials such as cereals, pulses, fruits, and vegetables, as well as fish and other marine products. LAB initiate relatively rapid growth in various plant materials over a wide range of temperatures and salt concentrations. A vast majority of Sri Lankans and Indians consume fermented traditional products as their breakfast and dinner. Yoghurt rice, "dosa", "idli" and hoppers are some examples of traditional cereal based fermented foods (Wickramanayake, 2002). *Idli* is a cereal and legume based fermented food, manufactured using LAB. Raw materials used for the preparation of *idli* batter are rice (*Oryza sativa*) and dehulled black gram (*Phaseolus mungo*). Following fermentation, *Idli* batter is steamed and formed into round shaped, white coloured, soft and porous textured product (Reddy *et al.*, 1982; Shetty *et al.*, 2005). Some of the LAB involved in fermentation may possess probiotic potential. Some studies have shown that *Idli* batter has LAB with probiotic potential. Those strains are *Lactobacillus collinoides* (Patel *et al.*, 2012), *Lueconostoc mesenteroides*, *Streptococcus faecalis*, *Pediococcus cerevisiae* (Mukherjee *et al.*, 1965),

Lactobacillus delbrueckii (Soni *et al.*, 1986) and *Lactococcus lactis*, *Lactobacillus plantarum* (Iyer *et al.*, 2011). The antibiotic sensitivity of probiotic microorganisms is an important characteristic determining their suitability for practical applications.

Considering the availability of probiotic potential LAB in *Idli* batter, their contribution to biochemical changes of *Idli* batter which improves the sensory quality of end product and therapeutic potential, the present study was conducted to isolate and characterize the novel strains from *Idli* batter and identify their antibiotic sensitivity.

METHODOLOGY

Black gram (*Phaseolus mungo*) and parboiled rice (*Oryza sativa*) were procured from local market in Vavuniya. All microbiological media and chemicals were obtained from HiMedia, India. The standard antibiotic discs were procured from Oxoid, UK. *Idli* batter was prepared by the procedure described by Wickramanayake, (2002). The black gram and parboiled rice were soaked in water separately for four to six hours. The seed coat of black gram was removed, mixed in the proportion of 1 to 2, mashed and left overnight.

Isolation of LAB

deMan Rogosa and Sharpe (MRS) agar medium was used for isolation of LAB as reported previously by De Man *et al.* (1960). The pH of the MRS medium was maintained at 6.2. *Idli* batter (1 g) was suspended in 9 ml of 0.9% saline water and subjected to serial dilutions. Diluted mixture (0.1 ml) was pipetted out and spread on the surface of an MRS medium agar plate. The plates were incubated in the inverted position for 48 h at 37°C under anaerobic conditions using candle jar technique.

Ten well developed characteristic colonies growing on the MRS medium were picked up carefully and purified by streaking on MRS agar medium plates following four-way streaking technique and subsequently purified for 4 to 5 times to get pure cultures. The discrete single purified colonies were picked up and transferred into MRS broth in culture vials and the grown-out cultures were maintained at 4°C in a refrigerator for further analysis (Pal *et al.*, 2005). To prepare fresh pure cultures for further analysis the preserved pure cultures were activated in MRS agar by streaking and incubated at 37°C under

anaerobic condition for 48 h before experimental use.

Identification and biochemical characterization

For pure cultures, Gram staining as described by Rangaswamy and Bagyaraj (1993) was carried out for morphological examination. Catalase activity (Gerhardt *et al.*, 1981), motility test (Barrow and Felthman, 1993) and spore forming ability (Barrow and Felthman, 1993) were used for biochemical identification of LAB from *Idli* batter.

In brief, the Gram characteristics of the isolates were determined for the LAB isolates grown 48 h using light microscope following staining. The Gram stained slides were observed under oil immersion (10x100) of light microscope and the cell morphology of LAB was observed as described by Becking (1974). Catalase test was done by transferring a loopful of LAB culture grown over 48 h into a glass test tube containing 0.5 ml distilled water and mixing thoroughly with 0.5 ml of 3% hydrogen peroxide solution. Subsequently, the effervescence was observed.

“Hanging drop method” was used to examine the motility of strains. At the centre of a coverslip the bacterial culture was dropped and each corner of the coverslip a drop of paraffin wax was placed. Over the coverslip a cavity slide was inverted to suspend the bacterial culture in the cavity slide’s central depression. Finally, the motility was observed under high-power dry objective (x40) with reduced illumination.

For endospore staining a thin smear of bacterial smear was prepared under aseptic conditions was formed on a microscopic slide, and heat fixed. Then the slide was placed over a steaming water bath and malachite green (primary stain) was applied for 5 min on the slide while covering the slide with blotting paper. Then the slide was removed from the water bath, the blotting paper was removed and the slide was rinsed with water until water turned clear. Then the slide was flooded with the counter stain safranin for 20 s and rinsed with water. Subsequently the slide was blotted-dried and observed using oil immersion lens.

Measurements during fermentation

The freshly prepared *idli* batter was allowed to ferment and during fermentation, samples of nearly 25 g were obtained from *idli* batter every 4 h interval up to 32 h and LAB counts were determined. The pH (Sension+ PH 31-Spain) and titratable acidity was determined every 4 h time interval up to 32 h of fermentation. After 4, 8, 12

and 16 h of fermentation, sensory attributes were determined based on a 5-point hedonic scale using a panel of 30 untrained panellists, who are familiar with *Idli* since childhood.

Evaluation of antibiotic susceptibility

The antibiotic activity of isolates was determined by Kirby Baeur disk diffusion method. All isolates were screened for their susceptibility to Amoxicillin (20 µg/disc), Erythromycine (15 µg/disc), Chloromphenicol (30 µg/disc) and Ciprofloaxacin (5 µg/disc). MRS agar was used as a basal medium for bacterial growth. Cell number of LAB isolates were adjusted by changing the absorbance (A_{600nm}) to 1 by using Phosphate Buffer Saline, pH 7.1 (10 mM Na_2HPO_4 , 1 mM KH_2PO_4 , 140 mMNaCl, 3 mMKCl) in order to standardize the number of bacteria (10^7 – 10^8 CFU/mL) and the cultures were spread on the surface of MRS agar using sterile cotton swabs. Above mentioned four standard antibiotic discs (Oxoid, UK) were placed on the inoculated plates with sterile conditions and incubated anaerobically at 37°C for 24 h. The diameter of the inhibition zone was measured following incubation. Breakpoints for the interpretation of inhibition zone was defined by expressed in terms of resistance (R), Intermediate (I) and susceptibility (S) according to the National Committee for Clinical Laboratory Standards, NCCLS (NCCLS, 1997).

RESULTS AND DISCUSSION

Well-developed characteristic presumptive LABs were isolated from *idli* batter. The characteristic colonies were identified on the surface of MRS agar Petri plate. Different type of colonies: Punctiform, small, moderate and large size colonies were observed on the surface. All colonies were non-pigmented in other words, creamy to white (Table 1).

The isolates were characterized further on the basis of their morphology, culture and biochemical characteristics named as phenotypical characteristics. Based on Gram staining, all isolates were Gram positive, 6 isolates were rod shaped while other 4 were cocci shaped. In case of endospore test, the isolates without green stained spores were termed non-spore formers. All cultures showed negative result thus they were identified as non-spore formers. The motility test has proven all isolates were shown non-motile behaviour. Catalase test is used to determine if bacteria possess catalase enzyme which can detoxify H_2O_2 . The LABs are negative to catalase

Table 1: Morphological characteristics of LAB isolates from *idli* batter

Isolate	Shape	Margin	Eleva.	Size	Texture	Appe.	Pigmen.	Opt. densi.
I-1	Circular	Entire	Raised	Moderate	Smooth, Moist	Shiny	Non-pig	Opaque
I-2	Circular	Entire	Raised	Large	Smooth, Moist	Shiny	Non-pig	Opaque
I-3	Circular	Entire	Raised	Puncti form	Smooth, Moist	Shiny	Non-pig	Opaque
I-4	Irregular	Entire	Raised	Moderate	Smooth, Moist	Shiny	Non-pig	Opaque
I-5	Circular	Entire	Raised	Small	Smooth, Moist	Shiny	Non-pig	Opaque
I-6	Spindle	Entire	Raised	Large	Smooth, Moist	Shiny	Non-pig	Opaque
I-7	Irregular	Entire	Raised	Small	Smooth, Moist	Shiny	Non-pig	Opaque
I-8	Irregular	Entire	Raised	Large	Smooth, Moist	Shiny	Non-pig	Opaque
I-9	Spindle	Entire	Raised	Moderate	Smooth, Moist	Shiny	Non-pig	Opaque
I-10	Spindle	Entire	Raised	Small	Smooth, Moist	Shiny	Non-pig	Opaque

Eleva. – elevation; Appe. – appearance; Pigmen. – pigmentation; Opt. densi. – optical density

test as they neither possess peroxidase enzyme nor produce bubbles when mixed with 3% hydrogen peroxide. Catalase test for all ten isolates was negative. This showed the absence of catalase enzyme and confirms that the isolates belong to LAB. From isolated 10 colonies, 6 isolates were found to be Gram positive, rods, non-motile, non-spore formers and negative to catalase activity, belonging to *Lactobacillus* spp. Other 4 isolates were Gram positive, cocci, non-motile, non-spore formers and negative to catalase activity, belonging to *Lactococcus* spp.

In summary, according to the above tests, it was confirmed up to the genus level that all strains were LAB and among them 6 were *Lactobacillus* spp (Table 2).

The main physiochemical characteristics of *Idli* batter fermentation is lactic acid formation. It can be noted by changes in the pH and titratable

acidity. Figures 1 shows the changes of titratable acidity and pH occurred during fermentation of *idli* batter. The measurements were taken at 4 h intervals from during 0-32 h period. During fermentation, titratable acidity increased after 8 h of fermentation of *Idli* batter from 0.24 to 0.32 % and the titratable acidity remained steady up to 20 h, and rose to 0.52% at 24 and 28 h, with a further rising at 32 h.

During fermentation of *Idli* batter, pH dropped steadily from 6.28 to 3.72 while titratable acidity increased from 0.24 to 0.92% during the period of 0 to 32 h of fermentation. Even though the fermentation temperature varied between 25 and 37 °C, the same observation has been recorded in several studies (Rajalakshmi and Vanaja, 1967; Soni and Sandhu, 1989; Soni and Sandhu, 1990; Ghosh and Chattopadhyay, 2012; Thakkar et al., 2015).

Table 2: Biochemical characterization of the LAB isolates from *idli* batter

Isolate	Gram reaction	Motility	Spore forming ability	Catalase activity
I-1	Gram positive, cocci in tetrad	Non motile	Nil	Negative
I-2	Gram positive, Rod shaped bacilli	Non motile	Nil	Negative
I-3	Gram positive, cocci in tetrad	Non motile	Nil	Negative
I-4	Gram positive, cocci in tetrad	Non motile	Nil	Negative
I-5	Gram positive, cocci in tetrad	Non motile	Nil	Negative
I-6	Gram positive, Rod shaped bacilli	Non motile	Nil	Negative
I-7	Gram positive, Rod shaped bacilli	Non motile	Nil	Negative
I-8	Gram positive, Rod shaped bacilli	Non motile	Nil	Negative
I-9	Gram positive, Rod shaped bacilli	Non motile	Nil	Negative
I-10	Gram positive, Rod shaped bacilli	Non motile	Nil	Negative

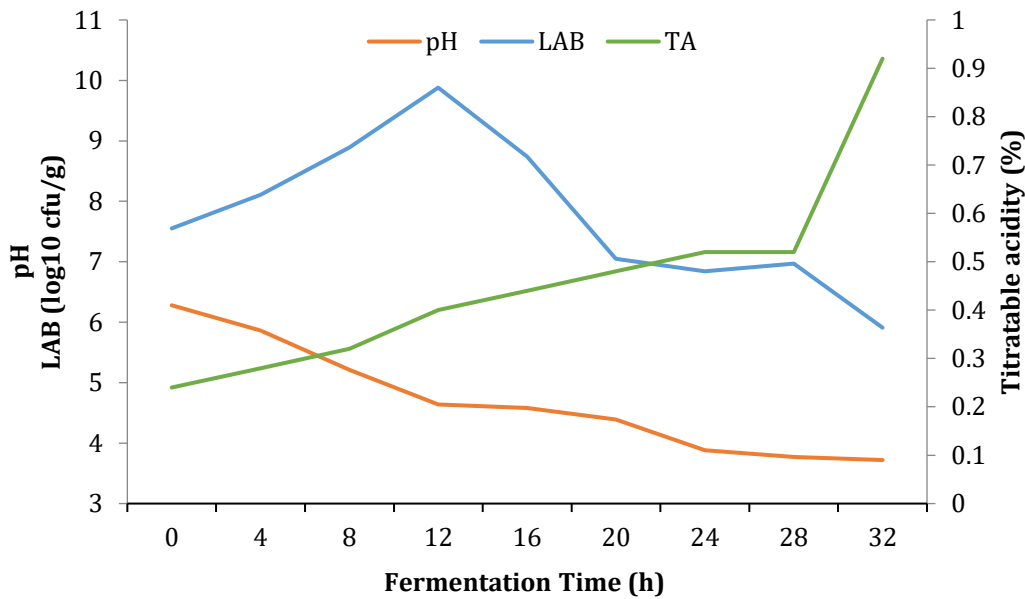


Figure 1. Change of pH, titratable acidity (TA) and lactic acid bacteria (LAB) count in *idli* batter during fermentation

The increasing acidity in *idli* batter mainly depends on *Streptococcus faecalis* as it produces lactic acid and reduces the pH, increases CO₂ concentration in the batter, which leavens batter property (Mukherjee *et al.*, 1965). With the growth of LAB, the pH of batter decreases (Figure 1). The reduction of pH of batter helps the activity of yeasts the optimum pH for yeast ranges from 4.4 to 4.5 (Soni and Sadhu, 1990).

The LAB count increased from 7.55 to 9.88 log₁₀cfu/ g during fermentation. High LAB count was observed after 12 h of fermentation of *idli* batter where the pH level was noted as 4.64 (Figure 1). The count was reduced with the increment of acidity. After 12 h of fermentation the LAB count dropped drastically from 9.88 to 5.91log₁₀cfu/ g during 32 h of fermentation. It may be due to increased acidity which may not favourable to LAB population.

The sensory quality of *Idli* prepared at different fermentation time intervals was analysed. The colour, appearance, taste, texture, mouth feel and the overall acceptability were taken into the analysis. The *Idli* prepared following 12 h of fermentation showed maximum desirable sensory quality where pH and acidity were 4.64 and 0.4%, respectively. *Idli* prepared with the batter

fermented for 4 and 16 h scored less for the overall acceptability. It may be due to the characteristic flavour and taste that may develop with prolonged

fermentation. Four hour of fermentation might have been not adequate to develop the desirable sensory attributes. Following 16 h of fermentation, higher acidity the sourness may be increased and the activity of microorganisms may cause undesirable texture of *Idli*. It may be the reason for the score of overall acceptability may be decreased (Figure 2). The maximum score of sensory quality was obtained at the LAB count noted high in number in the batter which was at 8 h and 12 h of fermentation time. Therefore, it indicates that LAB contribute to the textural and over all acceptability improvement of *Idli* batter. The same results were stated by different authors (Lee, 2001, Thakkar *et al.*, 2015) as when the pH and acidity randomly within the range of 4.0 - 4.5 and 0.5 - 0.6% at the time of fermentation showed optimum taste. One of the important *invitro* probiotic attributes that should be considered from the standpoint of safety before application of probiotic potential LAB in food that is antibiotic susceptibility. The fermented foods which have LAB may act as a pool to transfer antibiotic resistance genes to the pathogens through food or directly to the pathogens which are available in the gastro-intestinal tract while consumption of fermented foods (Lee *et al.*, 2012), (Coppola *et al.*, 2005; Toomey *et al.*, 2009; Erginkaya *et al.*, 2018). This study reports susceptibility profile of ten strains which were isolated from *idli* batter against different antibiotics such as Ciprofloxacin, Chloromphenicol, Amoxicillin and Erythromycin using the disc diffusion method. The inhibition zone was measured after 24 h of incubation.

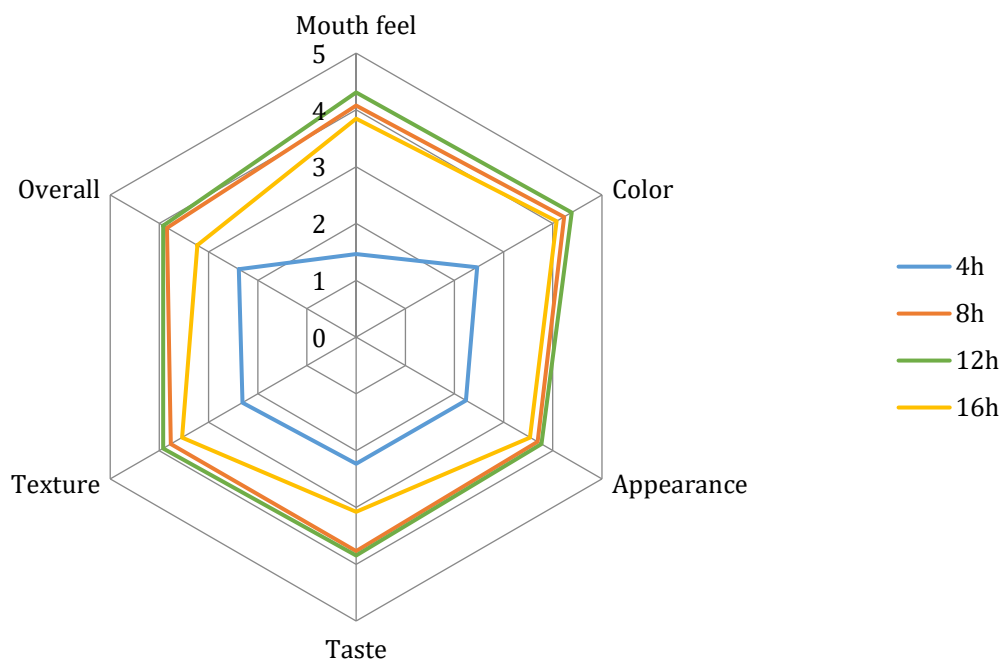


Figure 2. Web diagram depicting sensory attributes of *idli* prepared using different fermentation durations.

It was revealed that susceptibility to antibiotics highly varied among isolates. Most of the isolates from *idli* batter were sensitive to the major classes of antibiotics which are commonly used for therapeutic purposes and hence there appears to be a very low risk of possessing antibiotic resistant genes. It may be one of the reasons that the isolates are not more exposed to clinical antibiotics as they are wild strains. While, the commercially used LAB are more resistant to antibiotics as they regularly subjected to different drugs and environments during treatments (Erginkaya *et al.*, 2018), hence

they serve as pool of antibiotic resistant genes (Toomey *et al.*, 2009).

Among the antibiotics tested, Ciprofloxacin was effective against all isolates except I-1 and I-10, while Chloromphenicol usage was effective against all isolates other than isolates I-4 and I-8 (Tables 3 and 4). Similar findings have been recorded in previous studies that Chloromphenicol was sensitive to LAB isolated from *idli* batter (Patel *et al.*, 2012).

Table 3. Zone of inhibition shown by Lactic acid bacterial isolates against selected antibiotics

Isolate	Ciprofloxacin (5 µg/disc)	Chloromphenicol (30 µg/disc)	Amoxicillin (10 µg/disc)	Erythromycin (15 µg/disc)
I-1	8.3±0.58 ^b	15.3±1.53 ^{bc}	23.3±0.58 ^d	2.00±1.00 ^a
I-2	19.0±1.00 ^e	24.3±0.58 ^f	1.67±0.58 ^a	1.7±1.15 ^a
I-3	20.7±0.5 ^{ef}	21.7±1.15 ^{ef}	13.0±1.00 ^{bc}	24.7±1.15 ^e
I-4	23.3±1.53 ^f	12.3±1.53 ^b	28.3±1.53 ^e	21.7±0.57 ^e
I-5	11.3±1.53 ^c	17.0±1.00 ^{cd}	13.3±0.58 ^{bc}	6.3±1.53 ^c
I-6	20.0±1.00 ^e	24.3±1.53 ^f	2.00±1.00 ^a	5.33±0.58 ^{bc}
I-7	15.7±1.15 ^d	19.0±1.00 ^{de}	22.3±1.52 ^d	23.3±1.53 ^e
I-8	21.7±0.58 ^{ef}	4.33±0.58 ^a	11.7±1.15 ^b	2.33±0.58 ^{ab}
I-9	10.3±0.58 ^{bc}	15.0±1.00 ^{bc}	15.6±0.58 ^c	11.7±1.15 ^d
I-10	4.0±1.00 ^a	17.7±0.58 ^{cd}	23.7±1.15 ^d	13.3±1.53 ^d

Each value in the table was represented as mean ± SD (n = 30). Values in the same column followed by a different letter (a-b) are significantly different ($p < 0.05$).

Table 4. Profile of antibiotic susceptibility of LAB isolates

Isolate	Antibiotic susceptibility			
	Ciprofloxacin	Chloromphenicol	Amoxicillin	Erythromycin
I-1	R	S	S	S
I-2	S	S	R	R
I-3	S	S	I	S
I-4	S	R	S	S
I-5	I	S	I	R
I-6	I	S	R	R
I-7	I	S	S	S
I-8	S	R	I	I
I-9	I	S	S	I
I-10	R	S	S	I

R: Resistant, S: Sensitive, I: Intermediate

Among 10 isolates, 8 isolates namely I-1, I-3, I-4, I-5, I-7, I-8, I-9 and I-10 were susceptible to Amoxicillin while 7 isolates among 10 isolates namely I-1, I-3, I-4, I-7, I-8, I-9 and I-10 were susceptible to Erythromycin. In other words, Table 3 and 4 summarize the results of antibiotic susceptibility study.

Isolates I-3, I-7 and I-9 were susceptible to all antibiotics, while others were shown resistant to at least one antibiotic. The diameters of the zone of inhibition range from 1–23 mm. The highest zone of inhibition was observed by isolates I-6 against to Chloromphenicol (23 mm). While, the lowest zone of inhibition was observed by isolates I-2 against to Erythromycin (1 mm).

Adagbada *et al* (2012) have shown that antibiotics can create diarrhoea by creating gut micro flora imbalance and this can be prevented by probiotics. Resistance to antibiotics by probiotics is important in replacing the normal gut microflora after the

therapeutic treatment with antibiotics. It is recommended while using the LAB for starter culture it should be susceptible to at least to two antibiotics (Shuhadha *et al.*, 2017).

CONCLUSION

It can be concluded that Lactobacilli spp are the predominant LAB microbial group, involved in natural idli batter fermentation. It changes the biochemical characteristics of the idli batter, subsequently it improves the texture and organoleptic characteristics of final steamed product. Also, the isolated LAB were shown different sensitivity profile between the isolates against antibiotics. It acts as a barrier in the case of prepares a combination of therapy with antibiotics. While considering the usage of LAB from idli batter as a starter culture the multi antibiotics resistivity should be evaluated well to consider them as safe

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