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A study on isolation of antibiotic resistant probiotic lactic acid bacteria in dairy product (Dahi)

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Abstract

In view of commercialization of probiotic products to meet the growing demands of the consumer due to their proposed health benefits, these probiotics are used as food adjunct. In this milieu, probiotic organisms are considered to pool the resistant genes and transfer these to pathogenic bacteria. The present study contributed to the isolation of antibiotic resistance probiotic lactic isolates from the dahi sample. The dahi samples of 24 no's were collected from the different BBMP zones of Bengaluru. The results showed that the presence of lactococci, streptococci, leuconostoc and lactobacilli. Lactic isolates of 43 no's showed better activity in milk, acidity and DMC. The isolates further screened for probiotic property such as acid and bile tolerance by adjusting the pH to 2.0 and by using ox-bile of 0.3%. the 12 lactic isolates showed maximum tolerance to acid and bile genotypically identified as *L. lactis* ssp. *Lactis* (LL7) and *L. lactis* ssp. *Lactis* bv *diacetylactis* (LL10), among the 3 lactobacilli isolates *L. plantarum* (LB2), *L. fermentum* (LB5) and *L. helveticus* (LB24) in case of leuconostocs isolates as *L. mesentroides* ssp. *mesentroides* (LE1 and LE4) and all the streptococci as *S. thermophilus* (5). All the maximum acid and bile tolerated isolates further screened for antibiotic resistance property using E-strip method. Among 12 isolates tested only 5 lactic isolates (LL10, ST2, ST14, LE4 and LB2) showed resistance to commonly used antibiotics. The purpose of the study is to identify the antibiotic resistance probiotic lactic isolates.

Keywords: study on isolation, antibiotic resistant probiotic, bacteria, dairy product (Dahi)

Introduction

The bacteria are foremost life forms present on the earth. Many of them are beneficial for mankind among that lactic acid bacteria (LAB) constitute an important group of micro-organisms used in the fermentation of food. The presence and use of LAB in the traditional fermented foods indicate they have had the status of generally regarded as safe (Ouweland *et al.* 2002) [6]. The LAB also possess some of the health promoting benefit called probiotics. It helps in the maintenance of intestinal microbial balance and therefore it plays an important role in maintaining the health (Socol *et al.*, 2010) [7]. Recent reports indicate that increasing of research towards antibiotic resistance in LAB (Giraffa 2002) [4]. According to WHO the resistance to antibiotic is an ability of bacteria to survive the effect of inhibitory concentrations of antibiotics. When bacteria are exposed to the same antibiotic over and over, the bacteria can change and are no longer affected by the drug. Bacteria have a number of ways how they become antibiotic-resistant (Ammor *et al.*, 2008) [1]. Like other bacteria LAB can be antibiotic resistant and this might be a problem. LAB themselves are not pathogenic but they can transfer resistance gene to pathogenic bacteria that infect humans or animals (Teuber *et al.*, 1999) [8]. The antibiotic resistance in most of the typical LAB belongs to the G+C branch namely lactococcus, leuconostoc, lactobacillus, streptococcus, thermophilus and pediococcus. More specially fermented dairy products and meat and vegetable that are not heat-treated before consumption provide a vehicle for antibiotic resistant bacteria. Therefore the present study is focused on the isolation and screening of antibiotic resistant probiotic lactic isolates.

Materials and methods

Dahi Samples: The market dahi samples of 24 No's collected from 8 different BBMP zones of Bengaluru, 3 samples collected from each place and with the help of sterile sample bottles 100 g of sample were collected.

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Plating of Samples: Serially diluted the samples of dahi collected in sterile phosphate buffer. The first dilution was prepared by transferring 11 g of dahi to 99 ml of sterile phosphate buffer and thoroughly mixed. The required dilutions were prepared by using first diution for lactococci, leuconostoc and lactobacilli. The first dilution was subjected to laboratory pasteurization of 63 °C for 30 min. for the enumeration of streptococci and immediately cooled to room temperature and separately diluted for required dilutions. The 1ml of required dilutions were transferred to sterile labeled petri plates and molten agar medium maintained at 50 °C water bath poured into the sterile petri plates with 10-15 ml. M17 and MRS (De man rogosa sharpe) are the two medias were used to estimate lactococci (30 °C/24-48 hours) streptococci (37 °C/24-48 hours) and leuconostoc (30 °C/24-48 hours) lactobacilli (37 °C/24-48 hours) parameters respectively, mixed gently allowed to solidify and incubated anaerobically in a candle jar. By selecting the countable plates the colonies were counted and average count was expressed as cfu per g.

After enumeration of LAB the colonies were selected based on the morphology and transferred as well as maintained in respective broth media. Lactic isolates are purified and individually inoculated into sterile skim milk and incubated, milk setting time was noted and also tested for Titratable acidity (TA) and Direct Microscopic Count (DMC). The probiotic properties like acid and bile tolerance tests were carried out as per Rajashekar *et al.*, (2013), while the isolates genotypically identified from an external source such as MacroGen. Further the isolates were screened for the antibiotic resistance test phenotypically as per Charteris *et al.*, (2008) using 5 commonly used antibiotics by E-strip method.

Results and Discussion

The different brands of Market dahi samples of 24 no's were collected from 8 different BBMP zones of Bengaluru. The samples collected at low temperature brought to the laboratory for analysis of LAB. The findings shows that an average count of lactococci 4.89 log₁₀ cfu/g, streptococci of 4.48 log₁₀ cfu/g; Leuconostoc of 3.82 log₁₀ cfu/g and lactobacilliof 4.98 log₁₀ cfu/g, were obtained. The lactic acid bacterial isolates of 82 no's were obtained which is belonged to lactococci (LL1 to LL24) of 24 no's, streptococci (ST1 to ST15) of 15 no's, leuconostoc (LE1 to LE5) of 5 no's and 33 no's of lactobacilli respectively. The isolates further screened for curdling time and DMC. The 8 no's of lactococci set the milk with an acidity of 0.67%LA at 9 hr and DMC 7.28 to 7.34log₁₀/g and 8 no's of streptococci isolates took 6 hr with TA of 0.67%LA and DMC of 7.52 to 7.68 log₁₀/g counts. only 3 leuconostoc isolates set the milk at 14h with an acidity of 0.66% LA and DMC 7.59 to 7.71 log₁₀/g. The lactobacilli (8) isolates set at 8 hr with TA of 0.68% LA and DMC ranged 7.57 to 7.79 log₁₀/g.

Screening for acid and bile tolerance of the lactic acid bacterial isolates obtained from dahi samples

The acid and bile tolerance test are the two main test to assess the probiotic properties of lactic isolates such as lactococci, streptococci, leuconostoc and lactobacilli. The isolates showed less setting time, acidity and more DMC were selected for acid and bile tolerance test using M17/MRS broth. For acid tolerance test the pH of the broth is adjusted to 2.0. After 2 hours of incubation and immediately after inoculation were plated. The table 1 showed that only 2 isolates LL7 and LL10 maximum tolerated to acid. Incase of

5 streptococci isolates ST1, ST2, ST3, ST7& ST14 showed minimum reduction in their viable count. The leuconostoc isolates LE1 and LE4 showed more tolerance to acid. Where as 7 lactobacilli isolates are tolerated to low pH and most tolerated is LB2 followed by LB5 and LB 24 respectively.

Table 1: Acid tolerance of the selected lactic isolates

S. No	Type of lactic isolates	No. of Isolates	Isolate code	Acid Tolerance					
				0h	2h	CD(P< 0.05)			
1	<i>Lactococcus</i>	14	LL1	4.27 ^a	1.46 ^b	0.37			
			LL4	3.30 ^a	2.31 ^b	0.34			
			LL6	2.72	0	0.16			
			LL7	6.87 ^a	4.86 ^b	0.18			
			LL8	4.01 ^a	1.51 ^b	0.34			
			LL9	3.36 ^a	1.41 ^b	0.34			
			LL10	7.05 ^a	5.26 ^b	0.33			
			LL11	3.39 ^a	1.52 ^b	0.29			
			LL12	2.41 ^a	1.27 ^b	0.34			
			LL15	3.80 ^a	1.52 ^b	0.21			
			LL17	4.43 ^a	2.50 ^b	0.22			
			LL18	4.32 ^a	1.81 ^b	0.47			
			LL19	3.47 ^a	1.51 ^b	0.18			
			LL21	3.13 ^a	1.28 ^b	0.76			
2	<i>Streptococcus</i>	13	ST1	5.75 ^a	2.89 ^b	0.71			
			ST2	4.62 ^a	2.21 ^b	0.32			
			ST3	3.91 ^a	1.16 ^b	0.39			
			ST4	3.27 ^a	1.58 ^b	0.32			
			ST5	3.51 ^a	1.61 ^b	0.39			
			ST6	3.42 ^a	1.28 ^b	0.20			
			ST7	4.62 ^a	3.32 ^b	0.29			
			ST8	3.45 ^a	1.30 ^b	0.53			
			ST9	3.30	NIL	0.24			
			ST11	3.29 ^a	1.40 ^b	0.76			
			ST12	3.08 ^a	1.13 ^b	0.72			
			ST14	6.81 ^a	4.6 ^b	0.52			
			ST15	2.99 ^a	1.74 ^b	0.26			
			3	<i>Leuconostoc</i>	5	LE1	3.39 ^a	1.27 ^b	0.64
						LE2	3.55 ^a	1.87 ^b	0.61
LE3	3.25 ^a	1.27 ^b				0.35			
LE4	5.89 ^a	3.42 ^b				0.38			
LE5	2.97 ^a	1.37 ^b				0.46			
4	<i>Lactobacillus</i>	11	LB1	3.97 ^a	1.30 ^b	0.23			
			LB2	5.49 ^a	2.93 ^b	0.08			
			LB4	3.39 ^a	1.10 ^b	0.08			
			LB5	4.84 ^a	2.05 ^b	0.52			
			LB8	3.09 ^a	1.19 ^b	0.08			
			LB10	4.03 ^a	1.62 ^b	0.44			
			LB11	3.13 ^a	1.46 ^b	0.28			
			LB21	3.09 ^a	1.19 ^b	0.42			
			LB24	3.53 ^a	1.88 ^b	0.32			
			LB26	2.87	NIL	0.08			
			LB28	3.30 ^a	1.30 ^b	0.21			

Note: Similar superscripts indicate non-significant at the corresponding critical difference

Bile tolerance of the lactic isolates

All the 43 lactic isolates were examined for bile tolerance test using M17 and MRS broth containing 0.3% ox- bile. After an exposure of 6 hr the table 2 reveal that 2 isolates of lactococci LL7 and LL10 maximum tolerated to bile among 14 isolates. In case of streptococci isolates ST1, ST2, ST3, ST7& ST14 has more viable count out of 13 isolates. Only 2 leuconostoc isolates LE1 and LE4 showed maximum tolerance to bile among 5 isolates. Where as out of 11 lactobacilli isolates tested 7 isolates has maximum viable count after 6 hour of an exposure.

Table 2: Bile tolerance of the selected lactic isolates

S. No	Type of lactic isolates	No. of Isolates	Isolate code	Bile Tolerance		
				0h	6h	CD (P< 0.05)
1	<i>Lactococcus</i>	14	LL1	3.52 ^a	1.46 ^b	0.32
			LL4	3.28 ^a	2.31 ^b	0.30
			LL6	2.72 ^a	NIL	0.12
			LL7	6.81 ^a	2.93 ^b	0.19
			LL8	4.01 ^a	1.51 ^b	0.31
			LL9	3.22 ^a	1.08 ^b	0.37
			LL10	6.00 ^a	4.11 ^b	0.31
			LL11	2.52 ^a	1.11 ^b	0.27
			LL12	2.80 ^a	1.10 ^b	0.35
			LL15	3.34 ^a	1.38 ^b	0.27
			LL17	4.93 ^a	1.41 ^b	0.22
			LL18	3.84 ^a	1.45 ^b	0.49
			LL19	3.08 ^a	1.46 ^b	0.11
			LL21	3.13 ^a	1.28 ^b	0.73
2	<i>Streptococcus</i>	13	ST1	5.22 ^a	2.62 ^b	0.72
			ST2	5.24 ^a	2.00 ^b	0.32
			ST3	4.10 ^a	1.43 ^b	0.36
			ST4	3.26 ^a	1.58 ^b	0.32
			ST5	3.26 ^a	1.14 ^b	0.35
			ST6	2.73 ^a	1.12 ^b	0.22
			ST7	3.94 ^a	3.52 ^b	0.26
			ST8	3.12 ^a	1.13 ^b	0.52
			ST9	2.97 ^a	1.16 ^b	0.21
			ST11	3.21	NIL	0.79
			ST12	3.08 ^a	1.13 ^b	0.70
			ST14	6.18 ^a	3.39 ^b	0.58
			ST15	3.76 ^a	1.13 ^b	0.21
			3	<i>Leuconostoc</i>	5	LE1
LE2	3.10 ^a	1.10 ^b				0.67
LE3	3.07 ^a	1.27 ^b				0.32
LE4	4.90 ^a	2.48 ^b				0.36
LE5	2.70 ^a	1.07 ^b				0.42
4	<i>Lactobacillus</i>	11	LB1	4.27 ^a	1.28 ^b	0.21
			LB2	5.63 ^a	2.86 ^b	0.05
			LB4	3.68 ^a	1.15 ^b	0.08
			LB5	4.42 ^a	1.86 ^b	0.52
			LB8	3.09 ^a	1.19 ^b	0.04
			LB10	4.12 ^a	1.32 ^b	0.24
			LB11	3.13 ^a	1.31 ^b	0.35
			LB21	3.52 ^a	1.23 ^b	0.43
			LB24	3.23 ^a	1.46 ^b	0.28
			LB26	2.99 ^a	1.26 ^b	0.08
			LB28	2.92	NIL	0.19

Note: Similar superscripts indicate non-significant at the corresponding critical difference

The maximum acid and bile tolerated isolates were genotypically identified. Based on these results 2 lactococci isolates LL7 identified as *Lactococcus lactis ssp lactis* and LL10 was identified as *Lactococcus lactis ssp. lactis bv diacetylactis*. In case of 5 streptococci (ST1, ST2, ST3, ST7 & ST14) isolates were identified as *S. thermophilus*. Two isolates of leuconostoc LE1 and LE4 identified as *Leuconostoc mesenteroids ssp. mesenteroids*. Where as lactobacilli isolates LB2 was identified as *Lactobacillus plantarum* and isolate LB5 as *Lactobacillus fermentum* while LB24 was *Lactobacillus helveticus*. All the isolates identity were confirmed by 16S rRNA sequencing.

Antibiotic screening of the selected lactic isolates

In the present study the selected 12 probiotic lactic isolates were screened against the commonly used 5 antibiotics such as, gentamycin, penicillin, streptomycin, tetracyclin and vancomycin using the E-strip method described by the

Charteris *et al.* (2008). The isolates showed variable result with the different antibiotics, most of the isolates were susceptible towards most of the antibiotics. However, among two lactococci strains one was resistant to tetracyclin (LL10) and other was resistant to vancomycin (LL18) whereas two streptococcal isolates were resistant to penicillin and tetracyclin (ST2) and vancomycin (ST14). The only one leuconostoc isolate (LE4) showed resistant to both streptomycin and vancomycin while, among three lactobacillus isolates only LB2 had resistance to penicillin and tetracyclin. From the results obtained infers that antibiotic resistant is strain specific, species specific and antibiotic specific, this was true with present study also. Comunian *et al.* (2010) [3] reported that out of 121 strains *L. paracasei* isolated from Italian dairy and meat products treated with the antibiotics tetracyclin and erythromycin majority of *L. paracasei* originated from cheese were resistant to these antibiotics. Asharf and Smith (2016) [2] reported that, the seventeen lactic acid bacteria (LAB) and probiotic strains isolated from dairy based product showed resistance to acid, bile and antibiotic tolerance pattern using E-test. The *L. reuteri*, *L. rhamnosus*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, *Bifidobacterium lactis* and *S. thermophilus* were susceptible to ampicillin and erythromycin. Vancomycin and streptomycin tolerances were most common among species whereas tolerances for gentamicin, clindamycin and tetracycline were rare.

Table 3: Antibiotic screening of selected probiotic isolates

Sl. No.	Code	Minimum inhibitory concentration (mcg/ml)				
		GEN	PEN	STR	TET	VAN
1	LL7	6.0	0.75	24	1.5	8.00
2	LL10	4.0	0.50	8.00	NIL	2.00
3	ST1	1.50	1.50	96	0.75	3.00
4	ST2	0.50	NIL	1.50	NIL	1.50
5	ST3	8.0	0.19	12.00	6.00	2.00
6	ST7	0.75	4.00	1.50	24	16.00
7	ST14	4.00	6.00	16.00	4.00	NIL
8	LE1	1.50	0.25	24	8.00	6.00
9	LE4	3.00	1.50	NIL	16.00	NIL
10	LB2	4.50	NIL	48	NIL	4.00
11	LB5	6.00	1.00	64	8.00	1.50
12	LB24	1.50	25	24	2.00	8.00

Note: All the values are an average of 3 trials.

GEN: Gentamycin

PEN: Penicillin

STR: Streptomycin

TET: Tetracyclin

VAN: Vancomycin

Conclusion

In the present study the findings for the isolation of antibiotic resistant probiotic lactic acid bacterial isolates from dahi samples which have exhibited wide difference based on setting time, acidity, DMC acid and bile tolerance and also antibiotic resistance property. The study conclude that the lactic acid bacteria isolated from dahi sample showed acid and bile tolerance property exhibit the probiotic property. The probiotic isolates genotypically identified and further screened for antibiotic resistance property. The antibiotic resistance property is the most debated topic today because during chemotherapy, washing out of healthy bacteria along with pathogenic bacteria hence healthy bacteria should be resistance to antibiotic. There fore from the results obtained infers that antibiotic resistant is strain specific, species

specific and antibiotic specific, this was true with present study also.

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