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CHARACTERIZATION OF ACID AND BILE TOLERANT LACTIC ACID BACTERIA ISOLATES FROM THE DOMESTIC DAHI SAMPLES

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Abstract: Thirty domestic dahi samples were collected from different locations of Bengaluru and screened for lactic isolates. Out of 40 lactic nature isolates, 21 lactic isolates showed varied tolerance to acid and bile. The lactobacilli isolates were tolerated more, leuconostocs, streptococci moderate and lactococci least to low acid and 0.3% bile. All the acid and bile tolerated isolates were identified to their species level phenotypically and genotypically as *Lac.lactis ssp. lactis* (1no)and *Lac.lactis* ssp. *lactis* bv *diacetylactis* (1 no), among the 13 lactobacilli isolates *Lab.rhamnosus* (5 nos), *Lab.plantarum* (3 nos), *Lab.fermnetum* (3 nos), in case of leuconostocs isolates as *Leu. mesentroides* ssp. *mesentroides* (3 nos) and all the streptococci as *Str.thermophilus* (3 nos).

Key words: *Dahi, Lactic acid bacteria, Acid tolerance, Bile tolerance,*

1.Introduction: Lactic acid bacteria (LAB) are a group of micro-aerophilic, Gram-positive, non-spore forming organisms that ferment lactose to produce primarily lactic acid [1] Probiotics are live microorganisms thought to be beneficial to the host organism. According to the definition by [2] probiotics are "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". The gastric juice over two litres is secreted by parietal cells into the stomach each day [3] and it causes destruction of most of the microorganisms ingested by lowering the pH in the stomach to about pH 2.0-3.0 [4]. In spite of passage through stomach, probiotic bacteria must then survive duodenal transit, which is arguably less influential on bacterial viability than gastric transit [5]. The success of a probiotic organism also depends on the selected strain possessing bile tolerance characteristics [4]. The lactic acid bacteria like probiotics used in the preparation of various health deriving fermented milk products should tolerate to the acid and bile prevailing in the stomach of human beings. Hence, the lactic cultures isolated from the domestic dahi samples were tested for the acid and bile characteristics.

2. Materials and methods: Domestic dahi samples (100 g) were collected from the locations of Atturu, Chowdeshwarinagar, Byatarayanpura, Jalahalli**,** Jekkur, Kempegowdanagar, Kodigehalli, Mathikere, Vidyaranyapura and Yelahanka new town of Bengaluru. A total of 30 dahi samples, three from each location were collected for study.

2.1 Enumeration of Lactic acid bacteriafrom dahi samples: The dahi samples collected were serially diluted in sterile physiological saline. Dilution of the first was prepared by transferring 11 g of dahi to 99 ml of sterile saline and mixed thoroughly. Using the first dilution, required dilutions were prepared for lactococci, leuconostocs and lactobacilli. For streptococci, the first dilution was subjected to laboratory pasteurization of 63^0C for 30 min., cooled to room temperature immediately and separately diluted for required dilutions.The required dilutions of 1 ml weretransferred to labelled sterile petri plates, 10-15 ml of molten agar medium maintained at 50^0C water bath, mixed gently, inverted and anaerobically incubated in a candle jar after solidification as given below:

Isolation of lactic acid bacteria from dahi samples: The selected colonies of lactococci and streptococci from countable plates were transferred to M17 broth while colonies of leuconostocs and lactobacilli to MRS broth and incubated anaerobically at 30^0C and 37^0C for 24-48 h.After the growth was observed as turbidity in broth, the broth cultures were streaked on M17/MRS agar and incubated at 30 /37^0C. Single and discrete colony was selected and transferred to M17/MRS broth and incubated at 30/37^0C. After repeated purification, the purified culture was streaked/stabbed in agar slants, appropriately numbered and stored in deep freezer/refrigerator.

Acid tolerance: MRS/M17 broth after adjusting pH to 2.0 using HCl was inoculated with overnight cultures and incubated at 30/37^0C. Samples were drawn immediately and after 2 h of incubation and tested for the number of survivors by plating and incubating at 30/37^0C for 24/48 h **[6].**

Bile tolerance test: To 10 ml MRS/M17 broth containing 0.3% ox bile, were added with the 1% inoculum of lactic isolates and incubated. 1 ml of samples were immediately plated to determine the count at 0 h. Then the remaining broth was incubated further for 6 h and enumerated for survivors on MRS and M17 agar **[6].**

Phenotypic identification: All the lactic nature isolates were subjected for the detailed biochemical tests as per the standard procedure and identification given in the [7].

Genotypic identification: The phenotyped lactic isolates were subjected for genotypic by DNA extraction, and PCR by using universal primer meant for lactic acid bacteria.However, as these facilities were not available in the department it was suggested to get these parameters analysed from an external source such as Macrogen (South Korea).

Results and discussion: The lactococci count ranged from 3.39 to 6.41 with an average of 4.44 $\log_{10}$ cfu/g of dahi. In case of streptococci the counts were in the range of 3.29 to 4.16 with an average of 3.56 $\log_{10}$ cfu/g. Leuconostoc counts ranged from 3.06 to 3.87 with an average of 3.56 $\log_{10}$ cfu/g. In respect of lactobacilli the counts ranged from 3.45 to 6.06 with an average of 4.21 $\log_{10}$ cfu/g. It is generally observed that lactococci are the highest followed by lactobacilli. Both streptococci and leuconostocs were nearly the same in all these sample **(Table 1).**

Table 1: Enumeration of lactic acid bacteria of domestic dahi samples collected fro different locations of Bengaluru

Location code	Location	Mean viable count of Lactic Acid Bacteria, $\log_{10}$cfu/g ±SD			
		Lactococci	Streptococci	Leuconostoc	Lactobacilli
D1	Atturu	3.39±0.12	3.77±0.07	3.25±0.05	3.52±0.02
D2	Byatarayanapura	4.22±0.18	3.59±0.11	3.62±0.07	3.66±0.05
D3	Chowdeshwari nagar	4.37±0.48	3.38±0.08	3.36±0.16	3.45±0.52
D4	Jalahalli	4.62±0.67	3.37±0.14	3.54±0.06	3.88±0.24
D5	Jekkuru	4.05±0.09	3.39±0.08	3.48±0.15	3.99±0.04
D6	Kempegowda nagar	4.69±0.46	4.16±0.53	3.71±0.47	4.07±0.32
D7	Kodigehalli	3.89±0.20	3.43±0.10	3.46±0.11	4.19±0.11
D8	Marhikere	4.26±0.11	3.29±0.03	3.06±0.08	3.48±0.10
D9	Vidyaranyapura	4.49±0.20	3.86±0.23	3.77±0.26	6.06±0.51
D10	Yelahanka	6.41±0.03	3.38±0.12	3.87±0.39	5.75±±0.17
Average (SD)		4.44±0.79	3.56±0.28	3.56±0.28	4.21±0.93

From each location 3 samples were collected and plated using M17/MRS agar and incubated at 30 ^{0}C /37 ^{0}C
SD= Standard Deviation,

Predominance of certain group of LAB in dahi may be due to variations in ambient temperature and type of inoculum used. If the inoculum used were to be from the same house hold, the culture which grows rapidly will dominate over the others. Further, after the curd is set, if it is held at room temperature for long periods then the culture which with stands low acid conditions will dominate. On the other hand if dahiis held at refrigeration temperature after it is set, the culture which grew rapidly would dominate. In addition if the inoculum is from other sources such as commercial dahi the culture used in it will dominate.In a similar study by Pradeep (2007) has found the predominance of lactococci (7.82 $\log_{10}$cfu/g) followed by lactobacilli (5.45 $\log_{10}$cfu/g) and leuconostocs (3.20 $\log_{10}$cfu/g)in domestic dahi samples collected from Bangaluru.

Patel et. al [8] observed the predominance of lactobacilli fromdahi prepared from household buffalo milk of Gujarat and opined that abundance of lactobacilli could be due to their use as starter culture in the manufacture of buffalo milk based fermented milk products. [6] have reported predominance of leuconostoc of 4.54 log $_{10}$cfu/g followed by lactobacilli (4.20 log $_{10}$cfu/g), lactococci (3.71 $\log_{10}$cfu/g) and streptococci (3.22 log $_{10}$cfu/g) from 10 domestic dahi samples of different zones of Karnataka. [9] has reported the occurrence oflactococci,streptococci, leuconostoc and lactobacilli with a viable count ranging from 4.80 to 6.11log $_{10}$cfu/g from 4 dahi samples in Bengaluru.

Performance of isolates in sterile milk: All the 80 isolates were individually inoculated into sterile skim milk and incubated at 30/37^{0}C until milk got curdled. The time taken for curdling was noted and also tested for Titratable acidity (TA) and Direct Microscopic Count (DMC). It may be seen in **Table 2** that only 7lactococci isolates curdled the milk with a curdling time of 18-20h, TA of 0.64 to 0.70%LA and DMC of 7.45-7.60 $\log_{10}$ cells/g. In respect of streptococci only 8 isolates curdled the milk with a curdling time of 6 to 8 h, %LA of 0.45 to 0.55 and DMC of 7.59 to 7.92 $\log_{10}$ cells/g. Among all the leuconsotoc isolates tested only 5 isolates curdled the milk with a curdling time of 24h. These

isolates at the time of curdling showed an acidity of 0.62 to 0.64% LA and a DMC of 7.61 to 7.70log_{10}cells/g. Of the 40 isolates of lactobacilli tested, as many as 20 isolates curdled the milk in 18- 24h, with an acidity ranging from 0.66 to 1.0% LA and a cell count ranging from 7.31 to 7.95 log_{10} cells/g. In general it is observed that streptococci took the least time while leuconstocs took the highest time to curdle milk. Acid production as high as 1.0%LA was observed in lactobacilli isolates.

In general it was observed that majority of the isolates failed to set the milk when grown individually. Probable reasons could be that these cultures require an environment where in more than one culture is required for its growth exploring the associated interrelationships which normally exist under those conditions. Further these cultures were grown in sterile skim milk which is different from the boiled milk used in domestic dahi preparation.

Table 2: Performance of isolates in sterile milk

Group	No. of Isolate positive	Curdling time (h)	Titratable Acidity (%LA)	DMC (log_{10} cells/g)
Lactocooci*	LL2,LL4,LL5,LL6,LL9,LL12, LL15 (7 no's)	**18 to 20**	**0.64 - 0.70**	**7.45-7.60**
Streptococci**	ST2, ST3, ST4, ST6, ST8, ST10, ST14, ST15 (8 no's.)	**6 to 8**	**0.45 - 0.55**	**7.59 - 7.92**
Leuconostoc*	LE2, LE6, LE10, LE12, LE15 (5 no's.)	**24**	**0.62- 0.64**	**7.61-7.70**
Lactobacilli**	LB1,LB2,LB3,LB4,LB5,LB8,LB9,LB10,LB11,LB12,LB13, LB14,LB15,LB16, LB20,LB23,,LB24 ,LB26,LB29, LB30 (20 no's)	**18 to 24**	**0.66 to 1.0**	**7.31-7.95**

* Incubated at 30^0 C
** Incubated at 37^0 C

In a similar study carried out by [10] screened 10 isolates of LAB obtained from dahi samples for their activity in sterile skim milk and found that a small number of *S.thermophilus*, isolates set the milk at 24 h of incubation at 37^0C with DMC of 8.67-8.69 log_{10}cells/g with titratable acidity of 0.67-0.69% LA. Further it was reported that the number of leuconostocs and lactobacilli isolates curdling milk was also lower indicating that the field isolates failed to grow or tend to grow slowly when grown alone in milk. Rajashekar [6] have also noticed decreased growth and acid production by the field isolates of lactobacilli obtained from dahi samples across Karnataka state. Further they also observed that out of 105 isolates screened from the dahi samples collected from 10 zones of Karnataka, only 42 isolates set the milk at 18 h with the acidity ranging from 0.42-0.60 %LA and DMC ranging from 8.30-8.69log_{10}cells/g. Mahesh et. al [9] demonstrated that out of the 24 lactic isolates obtained from the dahi samples in Bengaluru, 13 isolates set the milk at 18 h with an acidity of 0.67%LA and DMC count ranging from 7.5 -8.25log_{10}cells/g.

Screening of isolates for acid tolerance: It may be seen in **Table 3** that out of 7 lactococci isolates tested only 2 isolates, LL2 & LL12, were able to survive in low pH with a marked reduction in their viable count which was reduced to 1.23 and 0.77 log_{10} cfu/g respectively from the initial count of 5.73 and 5.62 log_{10}cfu/g. In case of streptococci out of 8 isolates tested only 3 isolates, ST2, ST4 & ST6,retained good viable count. Similarly 3 leuconostoc isolates, LE2, LE6 &LE10, tolerated low pH condition out of 5 isolates tested. In contrast the majority of lactobacilli isolates(13), out of 20 isolates tested, tolerated low pH values and maximum survivors were seen in LB3 followed by LB9 with a viable count of 5.14 and 5.07 log_{10} cfu/g from the initial count of 7.21 and 6.23 log_{10}cfu/g respectively. In the present study out of 40 LAB isolates, which set the milk, only 21 lactic isolates showed resistance to the acid, other 19 lactic isolates did not tolerate the acid conditions after 2 h exposure at pH 2.0. Among the survived isolates variations were found. Species of the *Lactobacillus* exhibited acid tolerance, however, gastric resistance has been shown to be highly strain dependent, with great variations between strains of the same species [11] . In agreement with [11] the present study also showed that the *Lactobacillus* isolates had the highest tolerance to acid followed by streptococci, leuconostocs and the least survivability was by lactococcus isolates. Among the lactobacilli, two isolates, *Lab.rahmnosus*LB3 and *Lab. plantarum*LB9, showed the highest tolerance to the acid.

The findings in this regard confirm the observations of other investigators who also reported acid tolerance in *Lactobacillus* cultures [12]. The potential of the strains to withstand gastric conditions may be attributed to elevated enzyme ATPase activity which has been shown to be present in certain *Lactobacillus* strains. This enzyme increases a bacterium's tolerance to highly acidic conditions by generating a proton motive force across the cell wall, increasing the cell's intracellular pH when extracellular pH is low [13]. Apart from the *Lactobacillus* strains the three *Streptococcus* isolates, ST2, ST4 and ST6, also showed moderate resistance to acid at 2 pH, the reason might be due to the presence of EPS on the cells which may reduce the effect of acid shock to certain extent. This is in agreement with the findings of other researchers who also reported EPS producing cultures have shown acid tolerance, where as other observations also reported that the *Str.thermophilus* was most acid sensitive, but their presence stimulates growth of the probiotics by reducing the oxygen tension initially, producing growth factors and reducing the pH of the medium.The high EPS producing strains of *Str.thermophilus* showed a significant (*P*=.05) protective effect against to 2 pH [14]. The most vulnerable among the tested stains were lactococci, as they showed almost no growth after their exposure to acid environment, but their use along with probiotic culture may enhance the performance of the product by producing acid and flavour compounds. The most vulnerable among the tested stains were lactococci, as they showed almost no growth

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Screening of isolates for bile tolerance : It is important that probiotic cultures should tolerate bile in order to survive in the intestine. The bile salt tolerance of all the 40 isolates were tested in the presence of 0.4% bile salt after an exposure of 6 h. Out of 40 isolates tested only 19 isolates tolerated bile at pH 7.2 and the results are given in **Table 3**. Out of 7 lactococci isolates tested only one isolate LL12 mildly tolerated bile salt with the viable count reducing from the initial 5.62 to 2.00 $\log_{10}$cfu/g after 6 h of exposure to bile salt. Among 10 streptococci isolates tested only three isolates, ST2, ST4 &ST6 tolerated bile salt moderately with viable count reduced to 2.59, 2.93 & 2.56 $\log_{10}$ cfu/g from theinitial count of 4.59, 6.07 & 5.99 $\log_{10}$cfu/g respectively. Among 5 leuconostoc isolates tested only 3 isolates LE2, LE6 & LE10 showed tolerance to bile salt. In these isolates the viable count reduced from 5.53, 5.52 & 5.8 to 2.15, 1.93 &2.00 $\log_{10}$ cfu/g respectively. In the case of 20 lactobacilli isolates tested 13 isolates showed tolerance to bile salt. The maximum tolerance was shown by LB3 followed by LB9 while the lowest tolerance was exhibited by LB20 & LB23.

The significant (P=.05) reduction in survival of the strains observed after subjected for bile treatment, even though all the strains showed reduction in viable counts, the *Lab.rahmnosus* LB3 and *Lab.plantarum* LB9 were less affected during the bile exposure after 6 h. The variability in sensitivity/resistivity to bile conditions among strains may be attributed to differences in their Bile Salt Hydrolase (BSH) activity (an enzyme which de-conjugates and decreases the digestive capability of bile) as reported [4] . The findings of the study in this regard confirm the observations of several other researchers [15] who also reported sizeable variations in the bile tolerance among their probiotic strains. Maryam *et al*. (2009) have observed that *Lab*. *Plantarum* A7 and *Lab. Rhamnosus* GG showed superior growth rates when compared to the other strains tested,but after the addition of 0.3% (w/v) Oxgall, only *Lab.plantarum*A7 displayed the best growth ability. *Lab*. *acidophilus*H26 and *Lab*. *rhamnosus*L5K1 exhibited nearlythe same bile tolerance as that of *Lab*. *plantarum*A7.Another study by [16] on acid and bile resistance of lactobacillus isolates (4nos) obtained from probiotic dahi, showed that none of the four isolates resisted acid and bile conditions.

Table 3: Acid tolerance of the selected lactic isolates

Group	No. of Isolates	Isolate code	Acid Tolerance, Viable count log $_{10}$ cfu/g		CD (*P*=.05)	Bile Tolerance, Viable count log $_{10}$ cfu/g		CD (*P*=.05)
			Incubation time (h)			Incubation time (h)		
			0	2		0	6	
Lactococcus	2	LL2	5.73±0.04^a	1.23±0.15^b	0.35	5.73±0.04^a	0.00±0.00^b	0.018
		LL12	5.62±0.03^a	0.77±0.21^b	0.60	5.62±0.03^a	2.00±0.00^b	0.02
Streptococcus	3	ST2	4.59±0.02^a	3.30±0.15^b	0.80	4.59±0.01^a	2.59±0.01^b	0.05
		ST4	6.07±0.06^a	3.67±0.17^b	1.09	6.07±0.00^a	2.93±0.02^b	0.034
		ST6	5.63±0.31^a	3.32±0.08^b	0.23	5.99±0.01^a	2.56±0.11^b	0.18
Leoconostoc	3	LE2	5.24±0.23^a	1.30±0.26^b	0.3	5.53±0.03^a	2.15±0.17^b	0.29
		LE6	4.85±0.25^a	1.33±0.45^b	0.5	5.52±0.03^a	1.93±0.15^b	0.25
		LE10	5.23±0.00^a	1.53±0.24^b	0.22	5.8±0.20^a	2.00±0.20^b	0.82
Lactobacillus	13	LB1	7.32±0.28^a	4.42±0.63^b	0.52	6.78±0.53^a	4.34±0.20^b	0.09
		LB2	7.5±0.14^a	2.7±0.36^b	0.76	7.56±0.04^a	4.33±0.22^b	0.44
		LB3	7.21±0.09^a	5.14±0.49^b	0.43	7.21±0.09^a	5.59±0.15^b	0.28
		LB4	7.43±0.40^a	2.37±0.55^b	0.91	6.87±0.11^a	4.87±0.32^b	0.55
		LB5	7.13±0.06^a	2.42±0.15^b	0.42	7.10±0.10^a	4.10±0.10^b	0.23
		LB8	8.31±0.02^a	3.17±0.29^b	0.25	7.31±0.02^a	4.31±0.02^b	0.52
		LB9	6.23±0.06^a	5.07±0.00^b	0.29	7.25±0.13^a	5.27±0.15^b	0.07
		LB11	6.72±0.11^a	4.13±0.08^b	0.51	7.05±0.13^a	4.13±0.35^b	0.6
		LB12	6.35±0.00^a	3.87±0.32^b	0.38	6.82±0.16^a	4.87±0.21^b	0.42
		LB14	6.73±0.36^a	3.17±0.30^b	0.32	6.52±0.03^a	4.43±0.11^b	0.19
		LB15	6.01±0.22^a	3.93±0.15^b	0.56	6.09±0.08^a	5.14±0.00^b	0.16
		LB20	5.80±0.55^a	3.14±0.15^b	0.56	5.51±0.02^a	2.43±0.00^b	0.26
		LB23	5.60±0.00^a	2.77±0.00^b	0.38	5.60±0.06^a	2.40±0.00^b	0.38

Results are expressed as means ± standard deviation of means

abcd means with in a column without a common superscript are statistically significantly different(*P*=.05)

It was found in this study that*Lab.rhamnosus*LB3 and *Lab.plantarum* LB9 were found to be the most resistant strain to acid and bile conditions (2 log reductions). Similarly, other researchers have reported the ability of *Lactobacillus* strains to survive simulated gastric-intestinal conditions [17]. Rajashekar et.al (6) also carried out a similar study to find the probiotic nature of lactic isolates of domestic dahi samples of Bengaluru and found that only one isolate of*Str.thermophilus*out of 9 lactic isolates tolerated acid and bile, while on the contrary Mahesh (2014) has revealed that of 15 lactic isolates obtained from the 8 dahi samples only one isolate of each of*Lac. lactis* ssp. *lactis*, *Str.thermophilus*, *Leu.mesentroides* ssp. *mesentroides* and *Lab.fermentum* tolerated both acid and bile.Generally the probiotic cultures are ingested with a product which also may contain other ingredients including a natural prebiotic. The product containing protein, fat and prebiotic may coat the probiotic culture and action of both acid and bile on probiotics may be lesser, where as in the laboratory experiments for acid and bile, the lactic isolates are used directly without any other ingredients and hence the effect may be harsh on the isolates than when eaten with a product.

Pheontypic and genotypic characterization of acid and bile tolerant isolates: Two lactococcal isolates LL2 & LL12 identified as *Lactococcuslactis*,LL12 as*Lactococculactis* ssp. *lactis*bv*diacetylactis.* All the three isolates, ST2, ST4 & ST6, on subjecting them to a variety of identification tests revealed that they belong to *Streptococcus thermophilus.* All the three isolates, LE2, LE6 & LE10, to various tests it was found that they all belonged to *Leuconostoc mesenteroids* ssp. *mesenteroids* as they all possess typical characteristics of the species in particular dextran from dextrose and survival at 55^0C for 30min. In this study 13 *Lactobacillus* isolates found to possess the ability to tolerate acid and bile and the identity of all these 13 isolates was investigated. Based on these results five isolates LB1 to LB5, were identified as *Lactobacillus rhamnosus*, three isolates, LB8, LB11 &LB12, were identified as *Lactobacillus fermentum*, two isolates, LB20 & LB23, as *Lactobacillus delbruckii*ssp. *bulgaricus* and 3 isolates, LB9, LB14 & LB15 as *Lactobacillus plantarum. a*ll these 21 isolates identity was confirmed by 16 S r RNA sequencing.

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