

## **Antimicrobial Activity of Casein Fermentate of Probiotic Lactobacillus Spp.**

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### **Abstract**

Of the total 34 Lactobacillus cultures 12 were selected on the basis of maximum proteolytic activity. Among these Lb 24 and Lb 19 were highly proteolytic and Lb 63 was least proteolytic. All the 12 proteolytic Lactobacillus were assessed for probiotic attributes. Las 1, Lb 141 were having maximum resistance to acid at pH 1.0 for 3 h of treatment. La 1, Lb 19, Lb 22 and Lb141 were highly bile resistant at 1-2% level. Lb 25, Lb 63, Lb 141 and Lb 307 showed maximum cell surface hydrophobicity (i.e. 59.35-77.21%). Almost all the lactobacilli were resistant to vancomycin and sensitive to erythromycin, whereas, for other antibiotic variation in sensitivity was seen. Cell free supernatant of Lactobacillus was tested for antimicrobial activity and it was found that Lb 25, Lb 63, Lb141 and Lb288 were showing maximum antimicrobial activity. Sodium caseinate was prepared for fermentation and the fermentate was tested for antimicrobial activity against gram positive and gram negative bacteria, yeast and mold cultures. Lb 141 had maximum antimicrobial activity as compared to other Lactobacillus and selected for further studies. 100 µg / ml of the fermentate of Lb141 showed maximum antimicrobial activity against bacterial cultures whereas 120 and 150 µg / ml was inhibitory to mold and yeast, respectively.

**Keywords:** Antimicrobial activity, Bioactive peptides, Lactobacillus, Probiotic attributes, Proteolytic activity.

### **1. INTRODUCTION**

Milk naturally contains an array of bioactivities due to lysozyme immunoglobulin and growth factors. A number of bioactive peptides have been identified in milk proteins, such as casein and whey proteins, where they are present in encrypted form, stored as

pro-peptides or mature C-terminal peptides that are only released upon proteolysis or enzymatic digestion *in vitro* or *in vivo*. Role of lactic acid bacteria in generation of bioactive peptide during food processing is well known. Many of the Lactobacilli also established probiotic cultures. Probiotics are defined as “Live microbial food supplements beneficial to health and have a positive effect in the prevention and treatment of intestinal microbial balance (Kullisaar et al, 2002). The metabolic activity of the probiotic cultures also assist in the formation of biological active peptides from milk proteins such as casein and whey. Casein derived peptides have already found interesting applications as dietary supplements (phosphopeptides) and as pharmaceutical preparations (phosphopeptides,  $\beta$ -casomorphins) ((Korhonen and Pihlanto, 2006; Fadaei, 2012).

The present study was carried out in two phases. In the first phase, screening of standard Lactobacillus cultures for proteolytic activity and probiotic attributes was done. In the second phase, antimicrobial activity of sodium caseinate fermented by selected proteolytic, probiotic standard *Lactobacillus fermentum* Lb 141 was assessed.

## 2. MATERIAL AND METHODS

### 2.1 Microorganisms

**2.1.1 Lactobacillus Cultures:** Thirty two Lactobacillus Cultures obtained from National Collection of Dairy Cultures (NCDC), NDRI, Karnal (*Lactobacillus casei* spp. *casei* NCDC 17, 297, 298; *L. acidophilus* NCDC 14,16, 33, 195, 291; *L. brevis* NCDC 01; *L. helveticus* NCDC 05, 06, 288, 292; *L. delbrueckii ssp. Lactis* NCDC 03; *L. delbrueckii ssp bulgaricus* NCDC 08, 27, 277, 281, 293, 307, 308; *L. plantarum* NCDC 20, 21, 25, 221; *L. rhamnosus* NCDC 19, 24; *L. paracasei ssp. paracasei* NCDC 22, 63; *L. fermentum* NCDC 141, 156, 214) and two reference Lactobacillus Cultures (*L. johnsonii* La 1 MBU and *L. casei* Shirota MBU) obtained from Molecular Biology Unit (MBU), NDRI, Karnal were used in the present study

**2.1.2 Indicator organisms:** Six bacterial (*Staphylococcus aureus* MTCC1144, *Bacillus cereus* MTCC 1272, *Escherichia coli* 0157:H7, *Enterococcus faecalis* MTCC 439, *Bacillus subtilis* NCDC 70 and *Streptococcus pyogenes* VR114, five yeast (*Kluyveromyces marxianus* NCDC 39, *Torulopsis candida* NCDC 43, *Saccharomyces cerevisiae* NCDC47, *Saccharomyces cerevisiae* NCDC 50, *Rhodotorula glutinis* NCDC 51) and one mold cultures (*Rhizopus oryzae* NCDC 52) used in this study as indicator organisms were also procured from the National Collection of Dairy Culture (NCDC), NDRI, Karnal and MTCC (IMTECH) Chandigarh

### 2.2 Screening of lactobacillus cultures for proteolytic activity

32 standard NCDC Lactobacillus cultures alongwith two reference Lactobacillus cultures were used for screening of their proteolytic activity on milk agar and protease activity was determined according to method of Keays and Wildi (1970) with some modifications.

**2.3 Screening of lactobacillus cultures for probiotic attributes**

For selection of probiotic lactobacilli, the isolates were subjected to the tests recommended as per WHO standards. All the parameters (*In vitro* tolerance to pH and Bile Concentration, cell surface hydrophobicity, antibiotic susceptibility test and antimicrobial activity) were evaluated according the method described by Clark and Martin (1994).

**2.4 Antimicrobial activity of sodium caseinate fermentate**

Casein and Sodium caseinate was prepared by standard method in experimental dairy, NDRI, Karnal. Antimicrobial activity of sodium caseinate fermentate was determined by agar diffusion method and MIC was calculated.

**3. RESULTS AND DISCUSSION**

**3.1 Proteolytic activity of Lactobacillus cultures**

Thirty five percent of total Lactobacillus cultures showed strong proteolytic activity by producing zone of clearance in between 19-22 mm. whereas, remaining Lactobacillus spp. produced small zone of clearance (<8 mm). Tyrosine value indicates the extent of protein degradation to liberate peptides and amino acids by the proteolytic enzymes. Degree of proteolysis influences the release of bioactive peptides and amino acids during fermentation. Lactobacillus species could produce wide range of proteolytic enzymes. Proteolytic activity of probiotic organism to some extent may help in the gut to hydrolyse the complex protein moiety into easily digestible form of small peptides (Hayes et al, 2006). This assay confirmed that Lb 24 to be the most proteolytic strain with protease activity of 839 tyrosine units/ ml, followed by Lb 19 (785units/ml), Lb 141 (688units/ml), Lb 307 and Lb 22 (658units/ml).

**3.2 Probiotic attributes of Lactobacillus cultures**

Twelve selected proteolytic lactobacilli were subjected determining their probiotic attributes. Acid tolerance is one of the most important pre-requisites for the selection of probiotic lactobacilli as they must survive the harsh acidic environment in the gut to remain there for a while in good number and express their health promoting functions (Shah, 2007). Lb 19, Lb 141 and La1 were able to survive even at pH 1.0 for 3 hrs, whereas, Lb 33, Lb 63 and Lb 288 were able to survive upto 2 hrs at pH 1 and their acid tolerance was comparable to that of a standard probiotic cultures (Boylston et al, 2004) (Table 1).

**Table 2: Bile tolerance of Lactobacillus ssp**

Cultures	1%				2%			
	0h	1h	2h	3h	0h	1h	2h	3h
Shirota	0.168	0.157	0.173	0.147	0.178	0.181	0.7	0.173
LA1	0.236	0.283	0.316	0.447	0.223	0.277	0.321	0.467
LB19	0.135	0.167	0.19	0.221	0.203	0.227	0.244	0.248

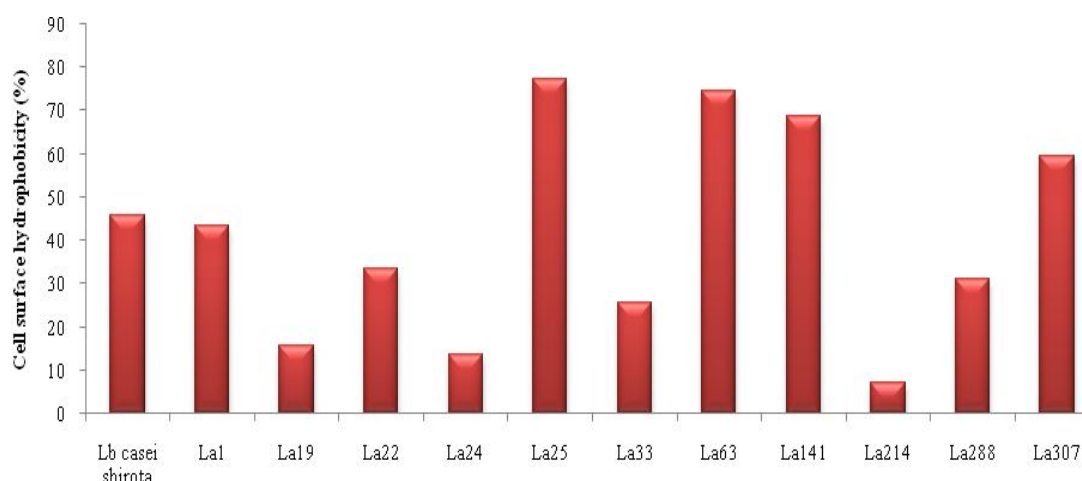
LB22	0.317	0.173	0.211	0.304	0.029	0.128	0.159	0.208
LB24	0.129	0.12	0.13	0.111	0.119	0.113	0.113	0.096
LB25	0.264	0.284	0.298	0.265	0.232	0.245	0.248	0.264
LB33	0.201	0.214	0.214	0.235	0.169	0.169	0.183	0.196
LB63	0.129	0.129	0.177	0.124	0.124	0.147	0.149	0.137
LB141	0.15	0.213	0.22	0.225	0.184	0.188	0.188	0.196
LB214	0.161	0.19	0.194	0.254	0.144	0.147	0.165	0.2
LB288	0.179	0.197	0.212	0.226	0.312	0.197	0.216	0.22
LB307	0.249	0.245	0.262	0.243	0.254	0.258	0.263	0.253

Bile tolerance is another important property used for selection of probiotic lactobacilli since human gastrointestinal tract (GIT) secretes high concentrations of bile in view of their potential role in food digestion ((Lankaputhra and Shah, 1997). In the present study, all the standard Proteolytic Lactobacilli tested were able to tolerate bile salt (1-2%) (Table 2). Attachment of bacterial cells to the intestinal mucosa is essential feature to impart beneficial effects on the host. Adhesion is the most important decisive factor for successful colonization. Hence, adhesion ability of probiotics to intestinal epithelial cell is considered as one of the most important selective criteria (Corthesy et al, 2007).. Lb 25 and Lb 63 were having maximum hydrophobicity (77.21 and 74.46% respectively) followed by Lb 141 (68.54%) and Lb 307 (59.35%) (Figure.1).

**Table 1: Acid tolerance of Lactobacillus ssp**

Cultures	pH1				pH2				pH3				pH6.5			
	0h	1h	2h	3h	0h	1h	2h	3h	0h	1h	2h	3h	0h	1h	2h	3h
Shirota	4.3	2.0	2.0	1.3	5.7 1	3.0	3.0	2.0	5.5	4.7 6	4.3	4	5.6	5.5 8	5.6	5.6
Lal	5.6 9	4.0 8	4.0 8	2.6 8	5.7 1	5.0	4.68	3.3 8	5.69	5.3	4.6	4.3	5.3	5.3	5.6	5.8 1
Lb19	5.3	2.3	2.3	1.2	5.3	2.8 5	2.78	2.0	5.48	5.5 3	4.0 8	4	6.5 8	6.6 6	6.8 4	6.8 9
Lb22	4.4 8	0.9	0.9	0	2.9	0.3	0	0	2.7	0.3	0.3	0.3	6.6	6.5 8	6.6	7.4 1
Lb24	0.9	0	0	0	1.4 8	0	0	0	4.4	3.7 8	3.4 4	3.4 8	6.6	6.5 8	6.6 6	6.8 9
Lb25	2	0.3	0.3	0	1.7 8	0.3	0.3	0	3.3	2.7 6	2.6	2.3	6.3	6.3 8	6.7 5	6.8 6

Lb33	1.7	1.4	1.4	0	1.2	1.1	1.0	0	5.78	5.4	4.4	4.4	6.4	6.4	7.4	7.
	7	7	7			8	9			8	8		8	8	8	6
Lb63	2.0	1.7	1.7	0	1.6	1.6	1.6	0.8	4.3	4.7	4.3	5.3	6.5	6.7	6.8	7.
	8	8	8							8			2	8	5	4
Lb141	5.4	3.4	3.4	2.3	5.3	3	3	2.7	5.78	5.8	3.3	2.0	5.9	6.0	6.5	7.
	8	5	5	8				8		5		8	5	1	2	1
Lb214	1.3	0.3	0.3	0	1.4	0.4	0.3	0	6.77	6.1	5.2	3.3	7.4	7.6	7.4	7.
					8	8				5			8		5	7
Lb288	5.5	2	2	0.3	5.6	3	2	1.9	4.07	3.7	2.4	2.7	5.3	5.3	5.6	5.
					4			5	9	9	8	9				7
Lb307	0.3	0	0	0	1.6	0.6	0	0	4.08	3.7	2.4	2.7	5.6	5.6	5.6	5.
					9	9				9	8	9			8	8
																1



**Fig. 1:** Cell Surface Hydrophobicity of Standard *Lactobacillus* Spp.

Antibiotic resistance/susceptibility of probiotic cultures can also influence their survival in the human gut since antibiotic therapy used to protect the GI tract can not only disturb the normal gut flora but also the probiotic cultures implanted therein (Sohail et al, 2012). Almost all the lactobacilli were resistant to vancomycin and sensitive to erythromycin (except Lb 25 and Shirota), whereas, for other antibiotic variation in sensitivity was seen.

Antimicrobial activity is yet another desirable potential attribute used for the selection of probiotic cultures. The ability to produce certain proteinaceous antimicrobial substances is an extremely beneficial property and hence can be used as a bonus to the probiotic culture to enhance its commercial value since the production of the antimicrobial factors by the probiotic culture in the gut can not only provide a

competitive edge to these beneficial cultures to survive and proliferate there but also help in the eradication of undesirable high risk pathogens and other infectious agents, the causative organisms of disease in human beings (Davis et al, 2007). Five cultures (Lb 25, Lb 63, Lb 141 and Lb 288) were able to show inhibitory activity against *B. subtilis*. The maximum antibacterial activity against *B. Subtilis* was shown by Lb 63, Lb 141 and Lb 288 while Lb 25 exhibited significant antimicrobial activity against *E. coli*, *B. subtilis* and mold cultures.

### 3.3 Antimicrobial activity of sodium caseinate of selected lactobacillus spp.

Sodium caseinate fermentate of Lb 141 showed highest activity against all the test cultures. Whereas, fermentate Lb 288 showed zone of inhibition against *B. subtilis*, yeast and mold but not against *E. coli*. However, Lb 63 and Lb 214 were inhibitory to *E. coli*. These results corroborate with Hayes et al, (2006) in which sodium caseinate fermented by *L. acidophilus* showed antibacterial activity against pathogenic *Enterobacter sakazakii* and *E. coli*.

### 3.4 Antimicrobial activity of sodium caseinate fermentate of L. Fermentum NCDC141

MIC of the sodium caseinate fermentate of Lb 141 was 100 µg/ ml against *E. coli*. 0.157: H7 and *B. subtilis* NCDC 70. For yeast this value is 150 µg/ ml and for mold the MIC was 120 µg/ ml these results corroborate with previous studies (Hayes et al, 2006).

## 4. CONCLUSION

The casein derived peptide could provide cheap, relatively safe, host stimulating antibiotics could be standardized by biological testing. Further research is needed to sequence the peptides. If the sequence of the peptides is known synthesis can be done commercially and use as an alternative to the antibiotics.

## 5. ACKNOWLEDGEMENTS

Priyanka Chandra is thankful to Science and Engineering Research Board (SERB), Department of Science and Technology, New Delhi (SB/YS/LS-112/2013) for financial support.

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