

Antimicrobial activity of sodium caseinate fermentate of *Lactobacillus fermentum* NCDC 141

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ABSTRACT

The sodium caseinate fermentated by *Lactobacillus fermentum* NCDC 141 showed antimicrobial activity against pathogenic strain *Escherichia coli* O157:H7 and against some spoilage causing microbial species *Bacillus subtilis* NCDC 70, *Saccharomyces cerevisiae* NCDC 47 and *Rhizopus oryzae* NCDC 52. This property of *Lactobacillus fermentum* NCDC 141 may have bioprotective applicability in milk based formula, which needs further exploration to confirm its potency.

Key words: Antimicrobial activity, Caseinate Fermentate, *Lactobacillus fermentum* NCDC 141, Proteolysis, Sodium.

INTRODUCTION

The aim of this study was to investigate the potential of *Lactobacillus fermentum* NCDC 141 to generate antimicrobial peptides from bovine casein and to assess its activities against pathogenic and spoilage microorganisms. The fermentate exhibited antimicrobial activity similar to that of antimicrobial peptide derived from *Lactobacillus casei* sub sp. *shirota* against strains of pathogenic or spoilage organism such as *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Rhizopus oryzae* which used in this study as a gold standard for activities against broad spectrum of microorganisms.

Lb. fermentum NCDC 141 was procured from National Collection of Dairy Cultures of National Dairy Research Institute, Karnal, India, and propagated in MRS broth (Himedia Ltd, Mumbai, India) for 48 h at 37° C. Similarly, *Escherichia coli* O157:H7, *Bacillus subtilis* NCDC 70, *Saccharomyces cerevisiae* NCDC 47, *Rhizopus oryzae* NCDC 52, procured from NCDC, NDRI, Karnal, India. *Lb. fermentum* NCDC 141 was selected for fermentation on the basis of its strongly proteolytic, probiotic potential and sodium caseinate fermentate antimicrobial capabilities demonstrated among the 12 different *Lactobacilli* species. *Lactobacillus fermentum* has been identified as potential probiotic strain (Mikelsaar and Zilmer, 2009) exhibiting adhesion to intestinal epithelia, inhibition of intestinal pathogens, bile salts resistance, intrinsically resistant to human gastric transit and as reaching the upper small intestine in high numbers after ingestion in the fasting state (Charteris *et al.*, 1998). The minimum inhibitory concentration of *Lb. fermentum* NCDC 141 fermentate was 100 µg/ml against

E. coli. The sodium caseinate fermentate from *Lb. fermentum* have not been reported previously as possessing antimicrobial activity. *Lactobacillus fermentum* ME-3 has also been found to have the capability to suppress mainly gram negative bacteria and to a lesser extent, suppress *Enterococci* and *Staphylococcus aureus* (Mikelsaar and Zilmer, 2009).

Bovine sodium caseinate fermentate generated using *Lactobacillus animalis* DPC6134 and *Lactobacillus helveticus* NCC 2765 showed angiotensin-converting-enzyme (ACE)-inhibitory activities (Hayes *et al.*, 2007). Bovine sodium caseinate fermentate peptides kill *Enterobacter sakazakii* ATCC 12868 spiked in reconstituted infant formula (Hayes *et al.*, 2009). Completely studied probiotic strain *Lb. casei shirota* has proven its potency in maintenance of healthy life and in prevention of diseases with daily consumption.

E. coli O157:H7 causes diarrhea and there are ample evidences that showed milk based infant formula and other milk based products have been served as a reservoir of infection. The possibility of using antimicrobial fragment of milk proteins such as sodium caseinate fermentate using *Lb. fermentum* NCDC 141 as an in built mechanism of protection against pathogenic strains such as *E. coli* O157:H7 raises the potential of producing a casein-based milk supplement. Antimicrobial peptides isolated from lactoferrin and α_{s1} casein having bactericidal, yeasticidal and antifungal activity against *E. coli*, *B. subtilis*, *Rhodotorula glutinis*, *Kluyveromyces marxianus* and *Candida guilliermondii*, *Aspergillus niger*, *Rhizopus oryzae* and *Penicillium* strains (Vij and Falguni, 2006).

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This study showed that suitability of *Lb. fermentum* NCDC 141 for production multiple antimicrobial compound from casein and particularly sodium caseinate fermentate may have bioprotective applications in milk based food products which is subjected to get confirm by further through investigation.

MATERIALS AND METHODS

The sodium caseinate substrate (2.5% w/v) was inoculated (1 %, w/v) with *Lb. fermentum* NCDC 141, incubated at 37°C for 48 h with 100 rpm and allow to get mix in triplicate. Culture was inactivated by heating at 80°C / 5 min and concentrated 10 folds by dehydrating at 50°C in incubator and concentration of protease enzyme was determined (Keay and Wildi, 1970).

Characterization of the antibacterial activity of fermentate involved measurement of growth inhibition in 96-well plate assay (Hayes *et al.*, 2009), while *Lb. casei subsp. Shirota* was used as positive control (Mikelsaar and Zilmer, 2009). The plates were incubated for 48 h at 37°C, and culture growth was monitored on hourly basis. MICs of the peptides were the lowest concentration without visible growth, measured by recording the optical density at 660 nm (OD 600) in spectrophotometer (Genova). A well diffusion assay was performed as described previously (Hickey *et al.*, 2003) using indicator strain *E. coli*, *B. subtilis*, *S. cerevisiae* and *R. oryzae* to detect antibacterial activity of concentrated peptides. The sensitivity of strain to fermentate was scored according to the zone of inhibition measured in mm. The fermentate was concentrated to 10 times exhibit antimicrobial activity. The positive controls used were the fermentate of *L. casei subsp shirota*.

The sodium caseinate fermentate produced by *Lb. fermentum* NCDC 141 when concentrated to 10 times by heating at 50°C possessed antibacterial activity however, antimicrobial activity was not detected in the fermentate, which was not concentrated or filtered through 10 kDa membrane, using well diffusion assay against any indicator strains. This may be due to low concentration of peptides. The concentrate fermentate exhibited the antimicrobial activity against *E. coli* O157:H7. The fermentate inhibited the indicator organism *E. coli* O157:H7 at a concentration of 100 µg/ml.

The MIC of fermentate was determined using control used with water added with no fermentate. *Lb. fermentum* NCDC 141 fermentate inhibited *E. coli* at concentration 100 µg/ml, where inhibition is defined as a reduction in growth rate of the test strains compared with no fermentate added against *E. coli* test strain.

Antimicrobial activity of sodium caseinate fermented:

Antimicrobial activity of sodium caseinate fermentate was determined for *Lb. fermentum* NCDC 141, by agar diffusion method (taking 10 fold concentrations) as described by Hayes *et al.* (2006) against *E. coli* O157:H7, *B. subtilis* NCDC 70, *Saccharomyces cerevisiae* NCDC 47 and *Rhizopus oryzae* NCDC 52.

Factors affecting proteolysis of sodium caseinate: to optimize the antimicrobial activity of sodium caseinate fermentate for antimicrobial activity different conditions were studied that includes temperature (30°C, 37°C and 42°C), incubation time (24 h, 48 h), pH (6.5, 7.0, and 7.5), substrates for fermentation (sucrose, lactose) and agitation (static, 100 rpm shaking incubator).

Bactericidal activity of *Lb. fermentum* NCDC 141 in sodium caseinate fermentate against gram negative and gram positive bacterial culture: Freshly grown cultures of *E. coli* and *B. subtilis* in Yeast extract Peptone Dextrose (YPD) broth (log phase), adjusted to 10⁴ cfu/ml were taken. *Lb. fermentum* NCDC 141 sodium caseinate fermentate was added to the broth based on MIC. *E. coli* and *B. subtilis* were incubated 37°C for 4 h. Optical density determined at intervals of 0, 1, 2, 3 and 4 hour at 660 nm (Genova spectrophotometer).

Fungicidal activity of Sodium caseinate fermentate against yeast and mold culture: Mold spores from YPD agar slant were added to 10 ml sterile saline water (0.8% NaCl, 0.1 % tween 80). Vigorously agitated, spore count was adjusted to 10⁴ cfu/ml using Haemocytometer. Sodium caseinate fermentate (10 fold) was added in YPD broth based on MIC. Freshly grown culture (log phase) of *Saccharomyces cerevisiae* NCDC 47 adjusted to 10⁴ cfu/ml were inoculated, incubated at 37°C for 4 h. Optical density determined at intervals of 0, 1, 2, 3 and 4 hour at 660 nm (Genova spectrophotometer).

Spectrum of inhibition of sodium caseinate fermentate of *Lb. fermentum* NCDC 141 against bacterial and yeast cultures: Test cultures such as *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus pyogenes*, *Enterobacter faecalis* and yeast culture *Rhodotorula glutinis*, *Kluyveromyces marxianus*, *Torulopsis candida* inoculated @ 2 % in YPD broth along with control, added 100 µg sodium caseinate fermentate of *Lb. fermentum* NCDC 141 per ml of YPD broth. Optical density taken at 660 nm at interval of 0 hour, 1 hour and 2 hour.

RESULTS AND DISCUSSION

Antimicrobial activity of sodium caseinate fermentate from selected *Lb. fermentum* NCDC 141: The sodium caseinate fermentate of *Lb. fermentum* NCDC 141 do not

exhibit antimicrobial activity after 24 and 48 h of fermentation. Sodium caseinate fermentate filter through < 10 KDa UF membrane did not show any zone of inhibition which may be due to less concentration of peptide in the fermentate. However 10 fold concentration of Sodium caseinate fermentate exhibit clear zone of inhibition (Table 1). It is clearly depicted in table 02 that sodium caseinate fermentate of *Lb. fermentum* NCDC 141 was highly antimicrobial against all the test cultures that is bacteria, yeast and mold.

TABLE 1: Antimicrobial activity of sodium caseinate fermentate (10 fold concentration) of *Lb. fermentum* NCDC 141 after 24 h incubation based on agar well assay method

Culture	<i>E. coli</i>	<i>B. subtilis</i>	<i>S. cerevisiae</i>	<i>R. oryzae</i>
<i>Shirota</i>	-	+	+	-
<i>Lb 141</i>	+	++	+	+

It is clear from the results obtained that *B. subtilis* was most; *E. coli*, yeast (Plate 1) and mold (Plate 2), were least sensitive to sodium caseinate fermentate. Similar results were obtained by Hayes *et al.*, (2006).

Studied different factors affecting proteolysis of sodium caseinate fermented of *Lb. fermentum* NCDC 141 fermentation at 37° C, pH 7.0, for 48 hour under shaking condition, 1% sucrose, 0.5 % lactose showed maximum proteolysis. Hence, these conditions were used for further analysis. (Table 2). Minimum inhibitory concentration of sodium caseinate fermentate was lowest for *E.coli* O157:H7 at 100 µg/ml. (Table 3).

Sodium caseinate fermentate of *Lactobacillus fermentum* NCDC 141 showed increase in O.D. after 4 hour of incubation, which might be due to growth stimulating

Table 2: Factors affecting proteolysis of sodium caseinate fermentate by *Lb. fermentum* NCDC 141

Parameters		Proteolytic activity units/ml	Optimum condition
Temperature ° C	30	84.66	37° C
	37	144.2	
	42	102.99	
pH	6.5	121.23	7.0 pH
	7.0	317.16	
	7.5	208.89	
Lactose	0.5	128.7	1.0 % lactose
	1.0	148.05	
	2.0	101.25	
Sucrose	0.5	277.2	0.5 % sucrose
	1.0	211.4	
	2.0	221.44	
100 rpm Shaking incubation	24 h	81.9	100 rpm for 48 h
	48 h	144.4	
No shaking incubation	24 h	88.29	48 h
	48 h	108.25	

TABLE 3: Minimum Inhibitory Concentration of sodium caseinate fermentate

Culture	MIC µg/ml
<i>E.coli</i>	100
<i>B.subtilis</i>	100
<i>S.cerevisiae</i>	150
<i>R.oryzae</i>	120

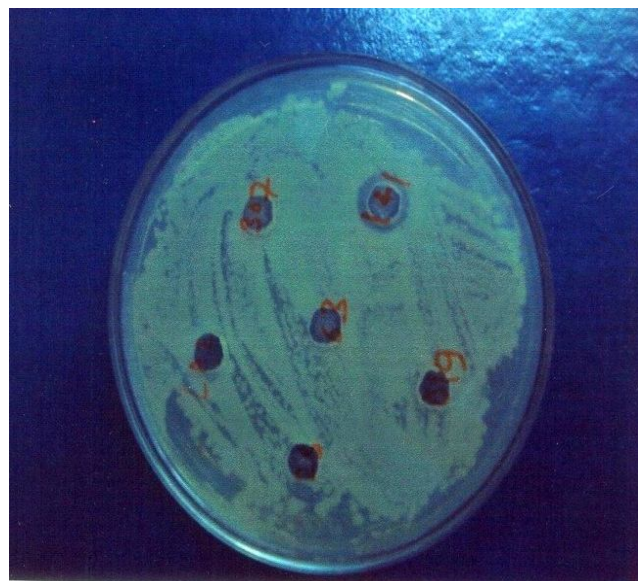


Plate 1: Antimicrobial activity of sodium caseinate fermentate produced by *Lactobacillus* species against *S. cerevisiae* NCDC 51 by 96 agar well assay method

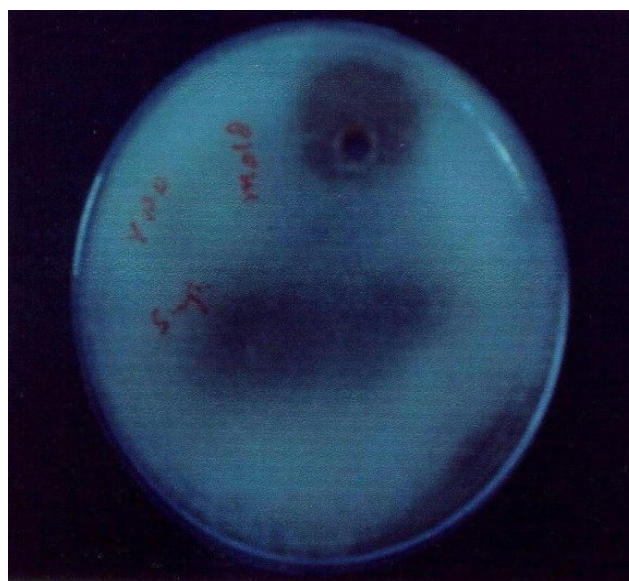


Plate 2: Antimicrobial activity of sodium caseinate fermentate produced by *Lb. fermentum* NCDC 141 against *R. oryzae* NCDC 52 by 96 agar well assay method

peptides release during fermentation, indicating lower bactericidal activity against *E. coli* O157 gram negative bacteria (Figure 01), and where as inhibitory effect depicted against, gram positive bacteria, *B. subtilis* NCDC 70 (Figure 2), yeast culture *S. cerevisiae* NCDC 52 (Figure 3) and mold culture *Rhizopus oryzae* NCDC 52 (Figure 4).

This result showed that, sodium caseinate fermentate of *Lb. fermentum* NCDC 141 was effective against gram positive bacteria, yeast, mold and less effective against gram negative bacteria.

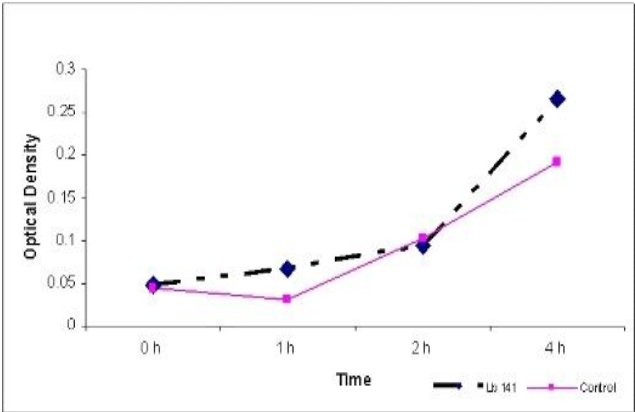


FIG 1: Antimicrobial activity of sodium caseinate fermentate produced by *Lb. fermentum* NCDC 141 against *E. coli* O157.

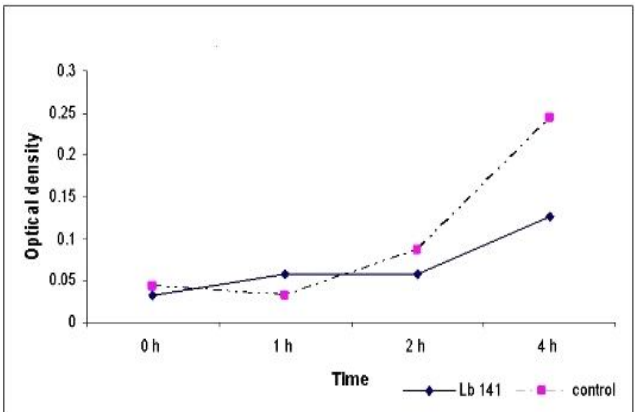


FIG 2: Antimicrobial activity of sodium caseinate fermentate produced by *Lb. fermentum* NCDC 141 against *B. subtilis* NCDC 70.

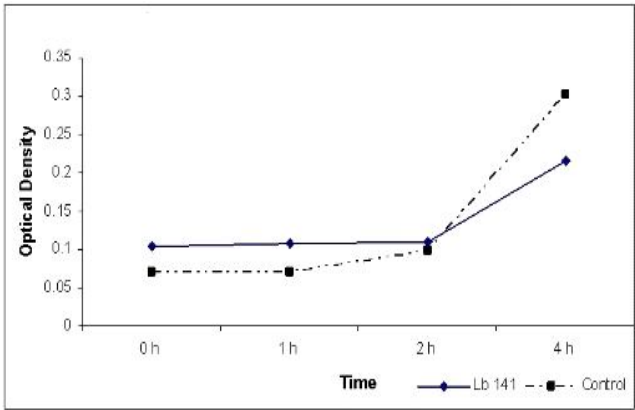


FIG 3: Antimicrobial activity of sodium caseinate fermentate produced by *Lb. fermentum* NCDC 141 against *Saccharomyces cerevisiae* NCDC 47.

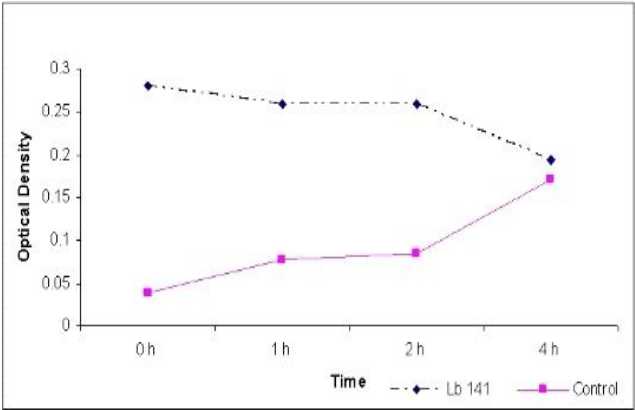


FIG 4: Antimicrobial activity of sodium caseinate fermentate produced by *Lb. fermentum* NCDC 141 against *Rhizopus oryzae* NCDC 52.

TABLE 4: Spectrum of antimicrobial activity of sodium caseinate fermentate produced by *Lb. fermentum* NCDC 141 against different bacterial cultures (result expressed as OD recorded).

Culture	<i>S. aureus</i>			<i>S. pyogenes</i>			<i>B. cereus</i>	
	0 h	1 h	2 h	0 h	1 h	2 h	0 h	1 h
Lb 141	0.040	0.100	0.131	0.055	0.076	0.051	0.101	0.084
Control	0.030	0.031	0.077	0.021	0.024	0.069	0.032	0.042

TABLE 5: Spectrum of antimicrobial activity of sodium caseinate fermentate of *Lb. fermentum* NCDC 141 against different yeast cultures (result expressed as OD recorded).

Culture	<i>Kl. marxianus</i> 39			<i>T. candida</i> 43			<i>R. glutinis</i> 50		
	0 h	1 h	2 h	0 h	1 h	2 h	0 h	1 h	2 h
Lb 141	0.030	0.0350	0.051	0.045	0.056	0.051	0.031	0.034	0.034
Control	0.030	0.031	0.077	0.021	0.024	0.069	0.032	0.042	0.078

inhibited, whereas *E. coli*, *E. sakazakii*, *L. bulgaricus* and *S. mutans* were inhibited by the test samples (Hayes *et al.*, 2006).

CONCLUSION

From the results obtained above, it is clear that sodium caseinate fermentate from *Lb. fermentum* NCDC 141

is effective against gram positive and gram negative bacteria, yeast and mold such as *Bacillus subtilis*, *E. coli*, *S. cerevisiae*, *R. oryzae*, respectively and could be use as a source of potential antimicrobial compounds when used in concentrated / standardized form against pathogenic microorganisms in milk and milk products.

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