Antibacterial Activity of Lactic Acid Bacteria isolated from Dairy Products against Enteropathogens

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Abstract
Lactic acid bacteria isolated from dairy products have received greater attention as potential food preservative due to their inhibitory activity against many food borne pathogens. In the present study, the antibacterial effect of 9 lactic acid bacteria isolated from milk (02), cheese (01) and curd (06) samples was investigated against five gram -ve bacterial isolates as Escherichia coli, Vibrio harveyi, Salmonella typhi and Klebsiella pneumonae by using agar well diffusion assay. The lactobacillus strain CC3 from curd sample showed maximum inhibitory activity against all selected test organisms. Growth profiles showed that maximum growth was observed at the end of late log phase and was accompanied with maximal reduction in pH of growth medium from 7.0 to 5.0. The strain CC3 was inoculated in soybean casein digest broth for four days and the cell free supernatant was assessed daily for production of bioactive compound.

It was observed that the isolate showed highest activity against all test organisms at the end of 72 hrs. This preliminary work shows the potential application of lactic acid bacteria in development of new therapeutic agents that may have role in preservation of fermented food.

Keywords: Enteropathogen, Antibacterial activity, Lactic acid bacteria.

Introduction

The balance and composition of the intestinal microbiota are crucial for maintaining gut health. A disease state may exist when this delicate balance between host-microbe relationship is distributed. The deviations in gut microflora are associated with risk of developing diseases such as irritable bowel syndrome, antibiotic associated diarrhoea and inflammatory bowel disease[13]. To increase the natural resistance of the host to such diseases, consumption of food containing live bacteria is most widely used method from ancient time. These beneficial organisms are termed as probiotics and affect host health positively by improving intestinal microbial balance[17]. Probiotic microorganisms are often incorporated in foods like yogurt, cultured milk products, infant formula s, breakfast cereals, sausages, chocolates and puddings. They are also available in the form of capsules and tablets[11]. The various probiotic preparations with positive therapeutic effects were used for treating traveller's diarrhea and antibiotic associated diarrhea[2] and also studied for attributes like antibacterial activity[6], antiviral activity[16], anti-yeast[12] and anti-oxidant activity[20]. The regulation of bowel activity and health benefits of probiotics is due to their more specific antagonistic effect on gastro-entero-pathogens like *Clostridium difficile*, *Campylobacter jejuni*, *Helicobacter pylori* and *Rotavirus*[10].

The antibacterial properties of protobiotic organisms may be due to production of bacteriocin[9], organic acids like lactic acid[22] or antimicrobial enzymes[19]. The bacteriocins like nicin are used in dairy products to prevent spoilage by Clostridial species in cheese. Some of the lactic acid bacteria like *Enterococcus*, *Lactococcus* and *Pediococcus* are also widely used as natural preservatives due to production of antimicrobial metabolite[14].

In the present study, the antibacterial activity of some lactic acid bacteria isolated from common dairy products like milk curd and cheese was assessed against enteropathogenic bacteria.

Material and Methods

Sample collection and culture: Four samples of milk (01), cheese (01) and curd (02) were collected in sterile containers and transferred to the Department of Microbiology, School of Life Sciences of Swami Ramanand Teerth Marathwada University, Nanded, INDIA. 1 mL of milk and curd samples was inoculated into 100 mL de Man, Rogosa and Sharp (MRS) broth medium (HiMedia, Mumbai) and cultured for 96 h at 37^0C 1 g cheese sample was macerated in sterile test tube containing 9 mL of sterile distilled water with the help of sterile glass rod. The macerated sample was filtered and the filtrate was used to inoculate in MRS broth and cultured for 96 h at 37^0C.

After incubation, the growth of lactic acid bacteria was observed in the form of turbidity and 0.1 mL of cultured broth of each sample was spreaded on MRS agar plates. The plates were incubated for 48 h at 37^0C. The well isolated morphologically distinct colonies were selected and transferred on MRS agar slants. A total of nine colonies, seven from curd samples and each one from milk and cheese sample were used for further studies. The isolated colonies were tested using gram staining, motility and catalase reaction. All nine isolates were gram positive and catalase negative.

Detection of antibacterial activity: Antibacterial activity of selected isolates was carried out by agar well diffusion assay[7]. The active cultures of isolates in MRS broth were inoculated in soybean casein broth supplemented with 0.5 %

dextrose and the flasks were incubated at 37℃ for 96 h. After incubation, the culture broths were centrifuged at 10,000 rpm at 4℃ for 10 min. The cell free supernatants were used as crude extracts and screened against enteropathogenic bacteria. The test bacteria used in this study are *E. coli, K. pneumonae, S. typhi, V. harveyi* and *V. parahaemolyticus.*

Active cultures of enteropathogenic bacteria in nutrient broth were diluted in sterile Mueller Hinton broth to reach the $O.D._{600}$ of 0.1. 0.1 mL culture was then individually spreaded on solidified Mueller Hinton agar plates. Wells of 5 mm diameter were punctured on the centre of plates with the help of alcohol sterilized cork borer and 30 µL crude extract of each isolate was added into the well. The plates were then kept in refrigerator for 30 min and all plates were incubated at 37℃ for 24 h.

After incubation, antibacterial activity was noted by observing the zone of inhibition around the well and the size of zone was measured to indicate the degree of inhibition. Streptomycin (50 $\mu g.mL^{-1}$) and uninoculated broth were used as positive and negative controls respectively. The isolate CC3 from curd sample showed good antibacterial activity and hence selected for further studies.

Growth kinetics and antibacterial compound production by strain CC3: Growth experiments were performed in Erlenmeyer flask of 250 mL containing 100 mL of soybean casein broth supplemented with dextrose (0.5%) at 37^0℃ under static conditions. An overnight active culture of CC3 in MRS broth was used to inoculate in soybean casein digest dextrose broth at initial $O.D._{600}$ of 0.1. At different time intervals, samples were removed from culture broth and used for optical density measurement at 600 nm, extracellular pH measurements and antibacterial compound production.

Results

Isolation of lactic acid bacteria: Nine bacterial strains isolated from curd, milk and cheese samples were found to be gram positive and non-motile rods. Seven isolates from curd were found catalase negative whereas two isolates each from milk and cheese showed positive catalase reaction (Table 1).

Antibacterial activity of lactic acid bacteria: These isolates were screened for their antibacterial activity against five enteropathogenic bacteria. The results of table 2 show that all nine isolates were active against *K. pneumonae* and *E. coli* and eight inhibited S. *typhi* whereas three isolates were active against *V. parahaemolyticus* and *V. harveyi.*

Table 1
Lactic acid bacteria isolated from dairy products

Isolate	Source	Gram's nature	Motility	Catalase reaction
CC1	Curd	G +ve rod	Non-motile	-ve
CC2	Curd	G +ve rod	Non-motile	-ve
CC3	Curd	G +ve rod	Non-motile	-ve
CC4	Curd	G +ve rod	Non-motile	-ve
CC5	Curd	G +ve rod	Non-motile	-ve
CC6	Curd	G +ve rod	Non-motile	-ve
CC6	Curd	G +ve rod	Non-motile	-ve
CC7	Curd	G +ve rod	Non-motile	-ve
MI1	Milk	G +ve rod	Non-motile	+ve
Cch1	Cheese	G +ve rod	Non-motile	+ve

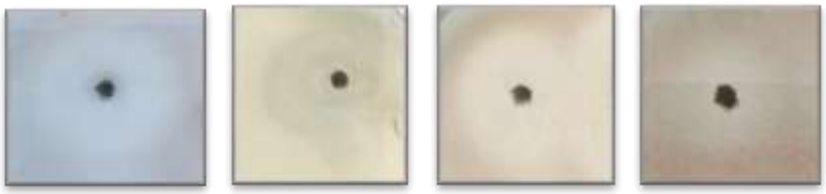
Table 2
Antibacterial activity of cell free supernatants of Lactic acid bacteria isolated from dairy products

Isolate	Test pathogen/ zone of inhibition (mm)				
	E. coli	***S. typhi***	***V. harveyi***	***V. parahaemolyticus***	***K. pneumonae***
CC1	-	36	17	14	18
CC2	-	28	-	-	19
CC3	22	18	15	13	18
CC4	-	25	-	13	13
CC5	-	14	-	-	17
CC6	-	-	-	-	27
CC7	-	12	-	-	19
MI1	-	15	10	-	19
Cch1	-	16	12	-	17
Streptomycin	-	23	25	24	21

Based on the sizes of inhibition zone and ability to inhibit all test pathogens, the isolate CC3 was selected as potent antibacterial producer strain (fig. 1).

Growth kinetics and production of antibacterial compound by isolate CC3: Growth and antibacterial compound production were studied in soybean casein digest dextrose broth at 37^{0}C and at pH 6.8. Under these condition growth, antibacterial activity and changes in pH of medium were detected periodically at an interval of 24 h. The results of the fig. 2 show that antibacterial activity increases with increase in cell growth as determined in terms of O.D.$_{600}$. The growth reached maximum between 48 and 72 h of incubation at 37^{0}C and declined thereafter. External pH of medium also started to decrease from second day of incubation and then declined further after 72 h incubation and remained constant till 96 h of incubation.

The isolate CC3 showed antibacterial activity against all test bacteria starting from 24 h of incubation which was increased further till 72 h and declined thereafter from 96 h of incubation.



(a) (b) (c) (d)

Figure 1: Antibacterial activity of CC3 isolate against (a) *S. typhi*, (b) *V. harveyi*, (c) *V. parahaemolyticus* and (d) *K. pneumonae*

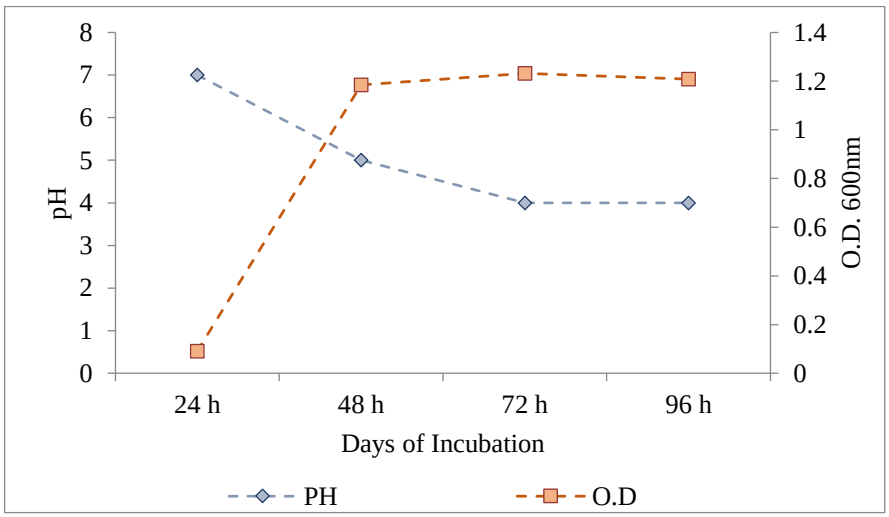


Figure 2: Change in pH and optical density during incubation period

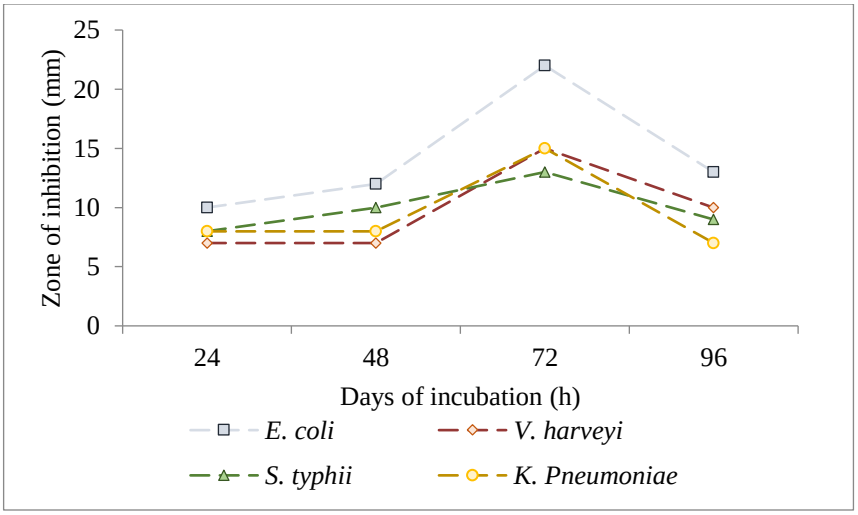


Figure 3: Antibacterial activity of CC3 isolate at varying time intervals

Table 4
Results of antimicrobial activity of supernatant collected at 3rd day

S. N.	Name of pathogen	Zone of inhibition
1	*E. coli*	22
2	*V. harveyi*	15
3	*S. typhi*	13
4	*K. pneumonae*	15

Discussion

Lactic acid bacteria are representative members of normal microflora and inhabit digestive tract of many animal species including human beings. Gastrointestinal infections are most common diseases caused by enteric bacteria including species *Salmonella, Vibrio, Shigella, Escherichia* and *Campylobacter*. Also, these bacteria have been identified as potential etiologic agents which cause severe illness in immunocompromised patients especially in human immunodeficiency virus infected patients[4,8] frequently causing malabsorption, significant weight loss, extra intestinal infections and increased mortality rates[4]. In this view, present study assessed the antibacterial potential of some lactic acid bacteria isolated from commonly used dairy products such as milk, curd and cheese against enteric pathogens.

Nine bacterial isolates from selected dairy products were identified based on their gram's nature, motility and catalase reaction. These phenotypic methods have been most commonly used for preliminary identification of lactic acid bacteria[6]. All nine isolates were gram positive and non-motile rods and showed catalase negative reaction except MI1 and Cch1 isolates. Lactic acid bacteria are G +ve, catalase -ve, non-spore forming, non-motile coccobacilli or rods and ferment glucose to lactic acid.

Lactic acid bacteria can produce antimicrobial agents that exhibit potent antagonistic activity against many spoilage causing and pathogenic microorganisms. The antimicrobial potential of lactic acid bacteria is due to their ability to produce metabolites such as organic acids, hydrogen peroxide, ethanol, acetone, acetaldehyde, reuterin and bacteriocins[21]. In the present study, all nine isolates inhibited growth of two or more test bacteria when evaluated by agar well diffusion method. The isolate CC3 was found promising due to its ability to inhibit all the test pathogens.

During growth studies, CC3 showed optimal growth after 72 h of incubation and was correlated with reduction in pH of medium and antibacterial activity. The initial pH of medium (6.8) was found to be reduced to 4.0 at third day of incubation and was same on fourth day. This indicated that maximum growth of CC3 was approached at third day during which it has fermented dextrose in the medium to produce lactic acid or acetic acid that dropped the pH of medium to 4.0. Organic acids produced by lactic acid bacteria reduce the pH levels and increase the production of hydrogen peroxide[18]. These products showed antibacterial effect against many pathogenic organisms including gram positive and gram-negative bacteria.[15]

In the present study, CC3 exhibited promising antibacterial activity against *E. coli, S. typhi, K. pneumonae* and *V. harveyi.* Many researchers indicated the antimicrobial potential of lactic acid bacteria isolated from various sources against *E. coli* and *Klebsiella sp*[3] and proposed that antimicrobial activity of LAB may be due to bacteriocin production.[1]

Conclusion

The results obtained in this study demonstrated significant antibacterial activity of LAB against enteric bacteria causing gastro intestinal infections. The antibacterial strain CC3 can be used as a best candidate for developing starter culture of products like curd and can be used for improving microbiological safety of traditional dairy products like curd.

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