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Effect of *Lactobacillus lactis cremoris* Isolated from Kefir against Food Spoilage Bacteria

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Abstract: The present study aims to control the food spoilage bacteria associated with food poisoning by LAB where it was isolated from kefir and identified as Lactobacillus lactis cremoris. Kefir is a delicious drink with probiotic activity. Nearly ten different food spoilage Bacteria's were isolated from spoiled food and it was used as test organisms. The susceptibility of test organisms towards the LAB was screened by the study of its effect of temperature, pH and Agitation. All the test organisms were labile to LAB. In this study, the LAB metabolite which is responsible for antibacterial activity shows its thermo tolerant even at 100°C for one hour. The activity of extract was very efficient at pH 4.5 and 6.5 and was ineffective at pH 8.5. The HPLC studies shows presence of bacteriocin and 68% was recovered. From this study we conclude that the lactic acid bacteria isolated from kefir which helps to control food spoilage and potential remedy to the food industries. The ability of the isolated LAB can produce heat stable as well as its acid tolerant which helps to prevent the contamination produced by endospore formers and other acid producing bacteria's.

Key words: Kefir, bacteriocin, zone of inhibition, LAB, food spoilage

INTRODUCTION

The 7th report (1993-1998) WHO Surveillance program for the control of food borne infection and intoxication in Europe has documented 5517 of outbreaks of food poisoning in Spain. In that period, nearly 69553 people were affected and 6820 people were hospitalized. In USA acute Gastroenteritis affects 250 to 350 million people annually and an estimated 22 to 30% of these cases are thought to be food borne disease. According to data from centre for disease control and prevention it has been estimated that approximately one in four Americans may experience some form of food borne illness each year. The bacterial pathogens that account for many of this case include *Salmonella*, *Camphylobacter jejuni*, *E. coli*, *Listeria monocytogen*, *S. aureus* and *C. botulinum* (McCabe-Sellers and Beattie, 2004).

To resolve the problem by means of controlling microbial spoilage by using LAB is an innovative microbial technique. LAB include the genera *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Cornebacterium*, *Aerococcus*,

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Oenococcus, *Vogacoccusand Weisella* (Hugas *et al*., 2002). These bacteria widely used as starter culture as well as they have wide range of antibacterial activity. The strategies for the application of LAB are diverse, inoculation of food with LAB, use of food previously fermented with the Bacteriocin producing strain or addition of purified bacteriocin in food. Most of the fermented milk products and their beneficial studies reveal that they have probiotic activity. Among these kefir is a widely used fermented milk product which is similar to curd (Wouters *et al*., 2002).

Kefir is a traditional popular Middle Eastern beverage. The world of kefir is said to have originated from the Turkish word Keyif which means good feeling. It is due to overall sense of health and well being when consumed (Chaitow and Trenev, 2002). It originates in the Caucasus Mountains in the former Soviet Union, in Central Asia and has been consumed for thousands of years. It is the product of fermentation of milk with kefir grains and mother cultures prepared from grains. Kefir grains look like pieces of coral or small clumps of cauliflower, which contain a complex mixture of both bacteria (including various species of lactobacilli, lactococci, leuconostocs and acetobacteria) and yeasts (both lactose-fermenting and non-lactose-fermenting) such that beneficial yeast as well as friendly probiotic bacteria found in yogurt. Kefir grains or mother cultures from grains (Libudzisz and Piatkiewicz, 1990) are added to different types of milk. It can be made from any type of milk; cow, goat or sheep, coconut, rice and soy but commonly cow milk is used. The grains cause its fermentation that results numerous components in the kefir including lactic acid, acetic acid, CO, alcohol (ethyl 2 alcohol) and aromatic compounds. That provides kefir's unique organoleptic characteristics: fizzy, acid taste, tart and refreshing flavor. Kefir possesses antibacterial activity in invitro against a wide variety of gram-positive and gram-negative bacteria (Serot *et al*., 1990) and some fungi (Cevikbas *et al*., 1994). Micro-organisms of genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Pediococcus* are involved in these fermentations. In addition, *Lactobacillus* sp. and species of *Bifidobacterium* which is not LAB in nature are part of normal human intestinal microflora and they exert a positive effect on human health (Daly and Davis, 1998).

MATERIALS AND METHODS

Nearly ten important food poisoning Microbes were isolated from spoiled food and used as test organisms against LAB, which was isolated from Kefir. The study period was October 2008 to January 2009.

Isolation of Food Spoilage Bacteria (Arakawa *et al*., 2008)

The Spoiled food samples like carrots, tomatoes and canned foods were used to isolate the food poisoning bacteria. About one gram of food sample weighed and serially diluted with PBS. One milliliter of 10^{-7} diluted sample plated Nutrient agar and PCA plates and incubated at 30°C for 24 h at aerobic and anaerobic condition. The isolated strains are subjected to biochemical tests.

Isolation of LAB from Kefir

The commercially obtained kefir starter culture was used to isolate the LAB. The kefir grains were inoculated on sterilized milk and incubated at 35°C for over night. About 1 g of Kefir serially diluted and one mL of sample from 10^{-6} was transferred into MRS medium for further identification by biochemical tests and compared with Bergey's manual of determinative bacteriology (Miller *et al*., 1997).

Effect of pH, Temperature and Agitation of LAB

The physiological parameters of isolated LAB were identified by using different pH, temperature and agitation. The M RS broth was prepared in three different pH (4.5, 6.5 and 8.5) where the temperature used in the studies are 10, 37 and 65°C and the agitation were at 75, 150 and 200 rpm. The growth rate of *Lactobacillus lactis cremoris* on different pH, temperature and agitation were observed at 360 nm.

Testing of Antibacterial Activity of *Lactobacillus lactis cremoris* Grown on Different pH (4.5, 6.5 and 8.5)

The antimicrobial activities of the isolates were quantified by modifying the disc-diffusion method assay procedure of Tadese *et al.* (2005). A well-isolated colony was selected from MRS agar plate culture. The top of the colony was touched with a loop and the growth is transferred into a tube containing sterile 50 mL MRS broth with three different pH. And the broth culture is incubated at 35°C for about 24 h. To get the culture filtrate a 24 h cultures were centrifuged (10,000 rpm for 20 min, at 4°C). The assay was performed against the test organism by well diffusion method.

Estimation of Total Protein (Lowry *et al.*, 1951)

The total protein was estimated by using Bovine serum albumin as a standard. Different concentration of standard solution was prepared and the Optical density of standard and test were taken at 360 nm (Lowry *et al.*, 1951).

Purification of Protein

Twenty four hours LAB grown MRS broth centrifuged at 10,000 rpm at 4°C to remove the cell. The supernatant were collected and equal volume of saturated ammonium sulphate was added and allowed to stand for overnight at 4°C. The mixture was centrifuged at 10,000 rpm at 4°C and the pellet was mixed with equal amount of 0.1 M Phosphate buffer saline and then dialyzed by using dialysis membrane at 4°C for overnight. The purified sample has been used against the test organism and its antibacterial activity was determined by the agar diffusion method.

Thermo Stability of Isolated Compound

The thermo stability of purified broth extract has been determined followed by incubating the extract in a three different temperature (60, 80 and 100°C) with different time intervals like 15, 30, 45 and 60 min. The thermo stability of the isolated compound was determined against *E. coli*. The thermally treated samples activity against test organisms was determined by the agar diffusion method.

HPLC

The bacteriocin was purified from a culture of *L. lactis* subsp. lactis grown in MRS medium at 30°C at pH 6.5. After 8 h incubation the culture was centrifuged for 30 min at 12 000 g, 4°C. The proteins were precipitated with 80% ammonium sulphate for 24 h at 4°C and centrifuged for 50 min at 17 400 g. The pellet was resuspended in 10 mL of 3 Murea and loaded on a Sep-Pack C 18 cartridge (Waters, Millipore). The cartridge was washed with 10, 40 and 80% acetonitrile. After drying under reduced pressure (Speed-Vac; Savant) the bacteriocin fraction was dissolved in 10% acetonitrile, 0.1% Trifluroacetic acid (TFA) in water. This fraction was used for final purification by reversed-phase perfusion liquid chromatography on a column PrepLC Universal Base Waters, RP-18 (120×25 mm, 15-20 μm)

on chromatography system (Biocad Sprint system, PerSeptive Biosystem, Voisins les Bretonneux, France). Bacteriocin was eluted with the following mobile phases: A (0.1% triuoroacetic acid in water) and B (0.1% trifluoroacetic acid in acetonitrile). Peptides were monitored spectrophotometrically at 220 and 280 nm, at a flow rate 20.0 mL min^{-1}. The fractions with highest bacteriocin activity were mixed and evaporated on a Speed-Vac concentrator (Savant). Eluted peaks were dried under vacuum, dissolved in deionized water and store at 20°C. Their protein content was estimated by the BCA Protein Assay Kit and their antagonistic activity was determined at each step of the purification process.

RESULTS

From the sample single isolated colonies were obtained and identified as *L. lactis cremoris* (Fig. 1). Its biochemical properties are listed on Table 1. the effectiveness of *Lactobacillus lactis cremoris* and its antibacterial activity toward the food spoilage test organism was identified by agar diffusion method. The isolated *Lactobacillus lactis cremoris* was effective against all ten test organisms. This indicates the extract having antibacterial component which is produced by *L. lactis cremoris*. The size of zone (>15 mm) indicates, among the ten test organism six are highly sensitive. The optimum temperature for the growth of *L. lactis* cremoris was at 37°C with 6.5 pH and 150 rpm (Fig. 2-4). Figure 2 shows the

Table 1: Identification of LAB isolated from kefir

Bio chemical test	*L. lactis* cremoris
Gram's staining	Gram positive, Irregular rod
Indole	-
Methyl red	+
Vogeus proskaur	-
Citrate	-
Catalase	-
Oxidase	+
Growth with NaCl	-
Glucose	+
Maltose	+
Lactose	+
Sucrose	-
Mannose	+
Xylose	-
Mannitol	-
Nitrate reduction	+

+: Positive, -: Negative

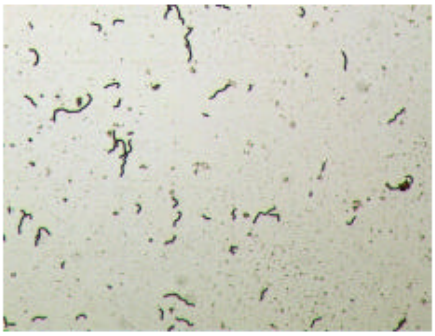

Fig. 1: Microscopic observation of LAB isolated from Kefir

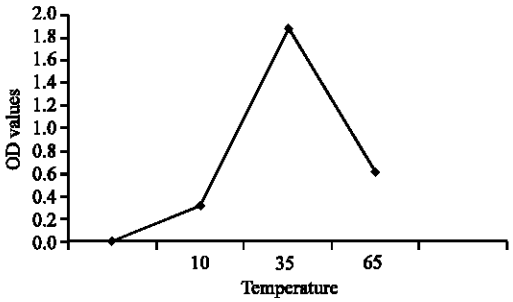

Fig. 2: Effect of temperature on growth of *L. lactis cremoris*

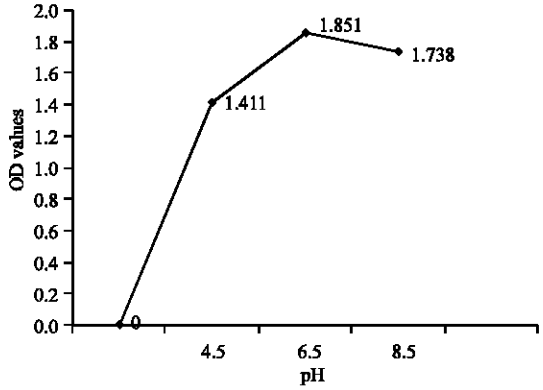

Fig. 3: Effect of pH on growth of *L. lactis cremoris*

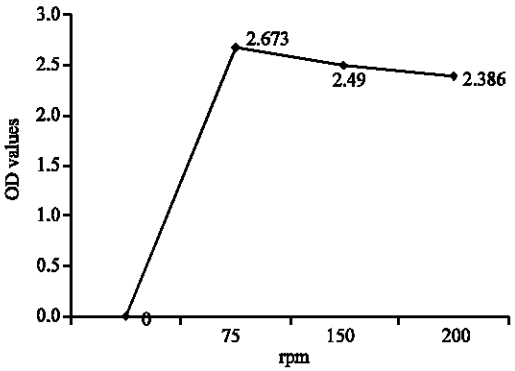

Fig. 4: Effect of Agitation on growth of *L. lactis cremoris*

maximum growth of *L.lactis cremoris* and the Optical density was 1.884. Where as the 10 and 65°C are shown 0.316 and 0.613 OD values. Figure 3 shows the optimum pH is 6.5, the LAB grown significantly at both the pH 4.5 and pH 8.5 but the activity of cell free culture filtrate is affected at the pH 8.5 not at 4.5. Figure 4 shows the agitation supports the growth of LAB. In this study we understand that the agitation could not affect the LAB growth.

The Food spoiling bacteria was controlled by the antibiotic produced by LAB which was grown on MRS broth. The total protein of the extract was higher in pH 6.5 and it was estimated as 30 μg (Fig. 5). The crude extract as well as purified extract exhibit antibacterial

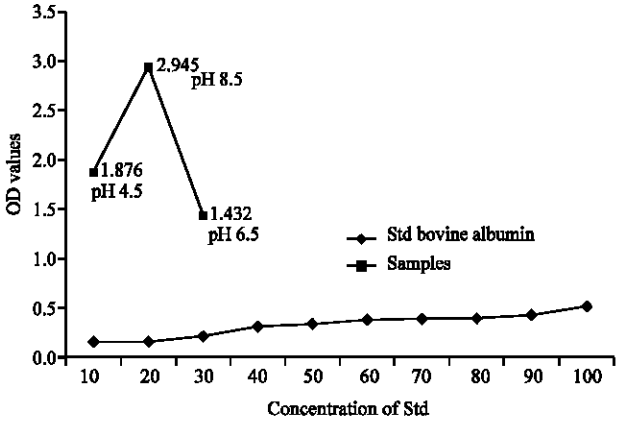

Fig. 5: Total proteins estimation of cell free culture filtrate

Table 2: Effect of pH on culture filtrate activity

	Size of zone formation (mm)		
Test organisam	pH 4.5	pH 6.5	pH 8.5
E. coli	20	33	Inactive
Pseudomonas sp.	11	13	Inactive
S. aureus	33	36	Inactive
Bacillus cereus	8	14	Inactive
Coliforms	12	16	Inactive
Klebsiella pneumoniae	10	14	Inactive
Proteus sp.	10	16	Inactive
Clostridium botulinum	13	15	Inactive
Fecalstreptococci	13	17	Inactive
Salmonella sp.	11	12	Inactive

Table 3: Determination of Thermo stability of purified extract of *L.lactis cremoris* grown on MRS broth

	Size of zone formation (mm)		
Test orgnisam	60°C	80°C	100°C
E. coli	32	33	33
Pseudomonas sp.	12.5	13	12
S. aureus	36	36	35
Bacillus cereus	12.5	14	13
Coliforms	16	16	16
Klebsiella pneumoniae	14	14	12
Proteus sp.	14	16	16
Clostridium botulinum	13	15	15
Fecalstreptococci	13	17	16
Salmonella sp.	11	12	12

property towards all the test organisms. The cell free culture filtrate retains it bioactivity even at 100°C for 1 h (Table 3). The activity of cell free culture filtrate shows restricted pH range. The activity is obtained at the neutral pH and acidic pH. However, the activity was slightly reduced at acidic pH (Table 2).

The studied bacteriocin was purified from 8 h culture in MRS at pH 6.5. The first step in the purification protocol was to concentrate the activity from the growth medium by ammonium sulfate precipitation. Approximately 1.5-fold concentration was achieved. The recovery was 68%. Further the precipitate was subjected to a Sep-Pack 18 cartridge. The active fraction was eluted with 80% acetonitrile. At this stage of purification, the recovery was 9.55% and the specific activity increased to 9850 AU mL^{-1}. This fraction was

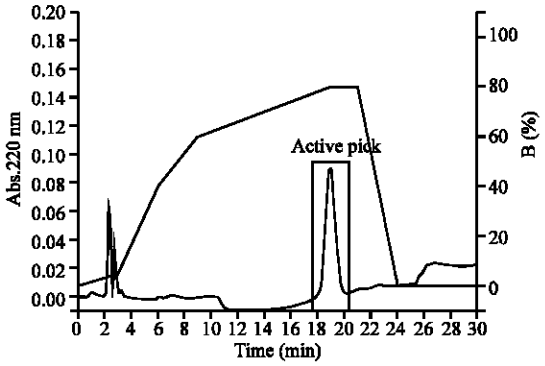

Fig. 6: Active fraction recovered from RP-HPLC eluted from Prep LC Universal BaseWaters, RP-18 column (120×25 mm, 15-20 μm) in same conditions. The Active peak was noted in the figure

Table 4: Purification of Bacteriocin produced by LAB

Purification	Volume (mL)	Activity (AU mL^{-1})	Protein (mg mL^{-1})	Specific activity (AU mg^{-1} protein)	Purification factor	Recovery (%)
Culture supernatant	500	6400	13.6	470	1	100
Ammonium sulphate precipitation, 80%	10	6400	9.3	688	1.5	68
Sep-pack RP-18 HPLC reverse phase RP-18	1	12800	1.3	9850	20.9	9.55
HPLC reverse phase rechromatography	0.200	12800	0.7	18285	47	5.14
	0.200	12800	0.6	21333	45	4.41

chromatographed on HPLC RP-18 reversed phase column. The eluted peaks were collected and checked for bacteriocin activity. At this stage the purification factor reached 47 and the recovery was 5.14%. The active fraction was purified by a subsequent reverse phase chromat ography. RP-HPLC chromatogram of the active fraction from Sep-Pack, a PrepLC Universal Base Waters and RP-18 column. The active fraction was noted in the Fig. 6. Rechromatographied active fraction recovered from RP-HPLC eluted from PrepLC Universal Base Waters, RP-18 column (Table 4).

DISCUSSION

The Lab isolated from kefir which is used in this study showed it maximum antibacterial activity at different pH and temperature. Application of this strain may helps to prevent the spoilage in food processing industries. Wide range of bacteria and fungi responsible for the spoilage of food but bacterial colonies are predominantly involved in food spoilage. The test organisms used in this study shows the gram positive and gram negative organisms and differ in their biochemical properties. Most of the lactic acid bacteria grow well under 6.5 pH. The utmost growth of *Lactobacillus lactis cremoris* were obtained at pH 6.5.

The spectrum of antimicrobial activity for the lactobacillus species suggested that the inhibitory components were different. Cadirci and Citak (2005) observed varying degree of inhibition of various food born pathogens by the culture filtrate of lactic acid bacteria, although these inhibitory substances produced by the lactic acid bacteria strains acts differently on the pathogenic reference indicator strains, inhibitive substances produced by the lactic acid bacteria can be generally protein. The strain specificity, the fact that the

prepared culture supernatants were neutralized as well the observation that even the non-neutralized supernatants had a slightly acid pH ranging from 5 to 5.5 suggested that the toxicity is not due to some low molecular weight organic or inorganic compounds. The antagonistic effects of kefir against *Salmonella kedougou* were attributed to the complexity and vitality of the kefir micro flora (Zacconi *et al*., 1995).

The *L. lactis cremoris* isolated from kefir and its growth on different pH such as 4.5 and 8.5 are moderate and at pH 6.5 was an opulent. The study on agitation and its growth at 75, 150 and 200 rpm were luxuriant. The LAB requires optimum temperature at 35°C and having the ability to withstand at 10°C as well as in 65°C. Mostly LAB having ability to utilize lactose and casein like protein in simple medium through their extracullular enzymes. the total protein of *L.lactis cremoris* at pH 4.5 and 8.5 comparatively less than 6.5 which shows thirty micrograms per milliliter. The total protein values of pH 4.5 and 8.5 are ten and twenty micrograms, respectively.

The antimicrobial property of the LAB grown on different pH shows the presence of antibacterial substance in the extract. The Best activity exhibited at pH 6.5 and 4.5 shows moderate activity, where as the pH 8.5 does not show any potential. Pediosin is a food preservative used in many industries which are thermo stable in nature. The LAB producing metabolite which is responsible for antibacterial property was very effective in all three different temperatures such as 60, 80 and 100°C. It denotes that the organism was capable to produce heat labile compound (Ahmed *et al*., 2004).

The use of LAB or Bacteriocins, either alone or in combination with mild physicochemical treatments will lower the concentrations of traditional and natural chemical preservatives, may be an efficient way to inhibit the spoilage and pathogenic bacteria. Certain LAB, with demonstrated antibacterial properties was commonly associated with foods, in order to prevent the food poisoning. There are many reviews were reported to prevent the food spoilage and food Spoilage pathogenic bacterial by Bacteriocin producing LAB. The use of Bacteriocin producing strains of LAB are of great interest as they are generally recognized as safe organisms for preventing the food poisoning as their antibacterial products which was used as bio preservatives. In the present study, it corroborates some of the *in vitro* studies of the bacteriocins and pediosin (De Vuyst and Leroy, 2007). The bacteriocin like substance was not active against *Escherichia coli.* All discovered sensitive strains were Gram-positive (Peeva *et al*., 2006). In this present study it was noted that the *Escherichia coli* is also sensitive to LAB. During purification several different protocols were applied (data not shown). Optimal recovery was achieved by including Ammonium sulphate precipitation and HPLC reversed-phase chromatography (Piard *et al*., 1990).

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