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Inhibitory Activity of Lactobacillus Strains against Food-Borne Pathogens

Nisha Goyal* and Krishnamoorthy Kannan

Department of Biotechnology, Guru Gobind Singh Indraprastha University, New Delhi-110078, India

**Corresponding author*

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A B S T R A C T

Since ages various physical and chemical food preservation methods have been to prevent microbial decomposition of food and thus preventing the food-borne illness. Most pathogens have become resistant to conventional methods of preservation and also the chemicals used are detrimental to human health. There is a need for nourishing, fresh and safe foods which can improve the health of consumer. The present research throws light to develop a probiotic based bio-preservative so that the food spoilage can be prevented by not causing various health hazards to the consumers. The present study was undertaken to check the inhibitory effect of different *Lactobacilli* strains namely *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* and *Sporolactobacillus laevolacticus*, if any, against various bacterial pathogens causing food borne illness such as *Salmonella typhimurium*, *E. coli* O157:H7 *and Bacillus cereus*. We found that the cell free supernatants of these *Lactobacillus* strains showed strong inhibitory activity against *S. typhimurium and E. coli* O157:H7 and a weak inhibition against spore forming *B. cereus*. Cell free supernatants of all the *Lactobacillus* strains showed inhibitory activity at acidic pH and lost their activity at alkaline pH, tolerated high temperatures even autoclaving and were sensitive to papain digestion (proteinase enzyme).

Introduction

There is a severe health burden on modern society in the form of various allergic and gastrointestinal diseases due to changed food habits and environmental pollution Foodborne illness is common in both developed and developing countries and continues to be a serious health hazard in developing countries leading to high morbidity and mortality rates. Food borne illness is mainly caused by ingestion of micro-organisms or their toxins in the food. Most common pathogens causing foodborne illness are *Salmonella typhimurium*, *E. coli*, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* (Nyenje and Ndip, 2013). *Salmonella* is one of the most common pathogen of food industry which causes food poisoning in humans. *Salmonella enterica serovar typhimurium* enters the food supply by contamination through beef, pork, poultry, dairy products and and nuts like peanuts and pistachios (Burkholder and Bhunia, 2009). *E. coli* infection spreads by coming in contact with faeces of humans and animals. The meat shows the presence of *E. coli* when it is not processed at 71 degrees Celsius. Also

unpasteurized milk and raw vegetables (lettuce, alfalfa, sprouts) are commonly contaminated with *E.coli* (Quinto *et al.,* 2014).

Bacillus cereus is associated with variety of food such as vegetables, meats, pasta, sauces, desserts and dairy products (Gordon *et al.,* 1973). *Bacillus* spores can survive at high temperature that is even after cooking food and germinates at the improper refrigerated condition. The food poison by *Bacillus cereus* is due to the production of toxins by this bacterium.

Resistance of these pathogens to conventional methods of food preservation and health hazards caused by chemical food preservatives has shifted the interest of Scientists towards development of safe and effective bio-preservatives developed from Lactic acid bacteria (LAB). Fermented foods and drinks are particularly rich in their content of LAB. LAB species of practical importance are usually nonpathogenic and safe. Several reports have indicated that the shelf-life of foods can be extended by LAB or LAB metabolites (Kalalou *et al.,* 2004; Schnürer *et al.,* 2005). Food safety is enhanced because of the inhibitory actions of LAB on common food-borne pathogens. Inhibition of food-borne pathogens by LAB can be mediated by competitive exclusion or production of organic acids and antimicrobial products such as hydrogen peroxide and antimicrobial peptides (e.g., bacteriocins) (McMULLEN and Stiles 1996; Ouwehand and Vesterlund 2004). These products can be produced by some LAB and secreted when grown in appropriate broth medium. Either live LAB or their metabolic products can be used. Thus, selected LAB species with these and additional qualities (termed probiotics, defined as live microorganisms which, when administered in adequate amounts confer health benefit on the host (Guarner *et al.,* 2005).

The present research focused on the elucidation inhibitory activity, if any, present in cell free supernatant of *L*.GG, *L.acidophilus* and *S.laevolacticus* against three pathogens causing food-borne illness namely *S.typhimurium*, *E.coli* O157:H7 and *Bacillus cereus.*

Significance and Impact of Study

This study elucidates the role of probiotics in inhibiting food borne pathogens. On the basis of this data further studies can be done to identify and purify the inhibitory molecule present in the Cell free supernatant of Lactobacilli and in future a bio- preservative can be developed against various food-borne pathogens.

Materials and Methods

All the bacterial cultures were procured from Microbial Type culture collection (MTCC), Institute of Microbial technology, Chandigarh, India

Growth Conditions

Salmonella enterica serovar typhimurium

Inoculate *Salmonella* from the fresh streaked petriplate into Brain Heart Infusion broth (5ml) and incubate at 37°C for 6 h on shaker. *Escherichia coli* O157:H7: Inoculate *E. coli* from the fresh streaked plate into 20ml of Luria Bertani Broth and keep it on shaker at 37°C for 8 h incubation. *Bacillus cereus:* Inoculate *B. cereus* from the fresh streaked plate into 20ml of Nutrient Broth and incubate it for 7 h on shaker at 30°C. *Lactobacillus acidophilus:* Inoculate *L. acidophilus* from the fresh streaked plate into 20ml of de Man Rogosa Sharpe broth and incubate it at 37°C for 48h *Sporolactobacillus laevolacticus:* Inoculate *S. lactobacillus* from the fresh streaked plate into 20ml of Glucose Yeast

Peptone broth and incubate it overnight at 35°C for 48 h. *Lactobacillus rhamnosus (L.GG)*: Inoculate *L*.GG from the fresh streaked plate into de Man Rogosa Sharpe broth 20ml and incubate it for 24 h at 37°C on shaker.

Preparation of Cell free supernatant of *Lactobacillus* stains

CFS of all the tested strains was prepared by centrifugation (10000 rpm/30 min/4 °C) of actively growing, overnight sub-cultured lactobacilli, followed by aseptic collection of the supernatant. The collected CFS was filter-sterilized by passing through a sterile Uniflo 0.2 µm pore size PVDF Whatman filter. *L*.GG-CFS labelled as CFSA, *L. acidophilus*-CFS labelled as CFSB and *S. leavolacticus*-CFS labelled as CFSC.

Agar well radial diffusion assay

Antimicrobial activity of CFSs of *Lactobacillus* strains against various pathogens were determined through modified agar-well diffusion assay (Presti *et al.,* 2015; Hassan *et al.,* 2011). Fresh overnight culture of each pathogen was streaked over respective media agar plate and incubated at 37 °C for 16 h. A minimum of four pure isolated colonies were transferred to sterile normal saline (0.85%) under aseptic conditions. Density of each microbial suspension was adjusted equal to that of 10^6cfu/ml (0.5McFarland standard) and used as the inoculum for performing agar-well diffusion assay. An aliquot of 100 µl of the inoculum of each test pathogen was spread plated over pre-solidified MHA plates. The inoculated agar plates were allowed to dry, and equidistant 8 mm wells were made using a sterile borer. The base of each well was sealed with molten agar medium. 100 µl of CFS was dispensed into each pre-labelled well and un-inoculated MRS broth (100 µl) served as negative control. In order to accelerate the diffusion of bacterial CFS into agar, MHA plates were pre-incubated at 4 °C/1 h, followed by overnight incubation at 37 °C. The antibacterial activity, indicated by the zone of inhibition (ZOI) surrounding the well containing the CFS, was recorded using zone scale (Hi-media).

All the tests were performed in triplicate, and the mean values of the diameter of inhibition zones were recorded. The inhibition continuums were recorded as follows: −, no activity (ZOI less than 1.1 cm); +, weak inhibition (ZOI of 1.2–1.5 cm in diameter); ++, moderate inhibition (ZOI of 1.6–1.9 cm in diameter); +++, strong inhibition (ZOI of 2.0–2.5 cm in diameter) and ++++, very strong inhibition (ZOI more than 2.5 cm).

Test of stability of inhibitory activity of cell free supernatant when exposed to different conditions

pH adjustment

The pH of the supernatant before pH adjustment was in the range of 4.5-4.8. A portion of each supernatant was subject to pH adjustment to acidic range 3-6 with 1N HCl and basic range 8-10 with 1N NaOH.

Papain treatment of supernatant

To test the sensitivity to proteases (purchased from Sigma), portions of each supernatant was incubated at 37°C for 1 h with and without papain (200 µg mL^{-1}) as papain is active over a wide pH range of 4-9 and the pH of *L*.GG-CFS is 4.5.

Heat Treatment of CFS

To test if inhibitory activity was heat-stable or sensitive, portions of each supernatant were heat-exposed to 60°C, 80°C and 101°C for 1 hr.

Lyophilization

Portion of each CFS was lyophilized also. CFS prepared as above was aliquoted and stored at -20°C. A fresh aliquot was thawed and used for experiments.

Statistical analysis

Results were expressed as mean ± SD.

Results and Discussion

Although the use of LAB as probiotics has received well attention, the application of LAB to reduce food poisoning cases is still underexplored. The infection of foodstuff with foodborne and pathogenic bacteria are global issue and it is severe hazard for the health of the human (Muhialdin *et al.,* 2012). This study aimed to explore inhibitory activity of *Lactobacillus* strains against various food-borne pathogens

Cell free supernatants were prepared from lag phase, log phase and stationary phase grown *Lactobacillus* cultures. It was observed that the inhibitory activity of CFS of all the three tested *Lactobacillus* strains was initiated in the log phase and peaked in the stationary phase. Hence, for all the experiments CFSs were prepared from 24h grown *Lactobacillus* cultures. Zones of inhibition against *S. typhimurium* were observed at 40, 50, 60, 70ul of CFSs. However, 60 ul was found to be the optimum volume where the inhibitory activity was maximum showing a clear zone. CFSA showed the strong inhibition with zone of 2.0±0.2cm, CFSB showed the moderate inhibition with zone of 1.6±0.3cm and CFSC showed weak inhibition with zone of around 1.2±0.2cm. Figure 1 shows the ZOI on MHA agar plates.

Zones of inhibition of *E. coli* were observed at 40, 50, 60, 70ul of CFSs. However, 70 ul was found to be the optimum volume where the inhibitory activity was maximum showing a clear zone. CFSA and CFSB showed the strong inhibition with zones of 2.0±0.2cm whereas CFSC showed moderate inhibition with zone of around 1.6±0.2cm. Figure 2 shows the ZOI on MHA agar plates.

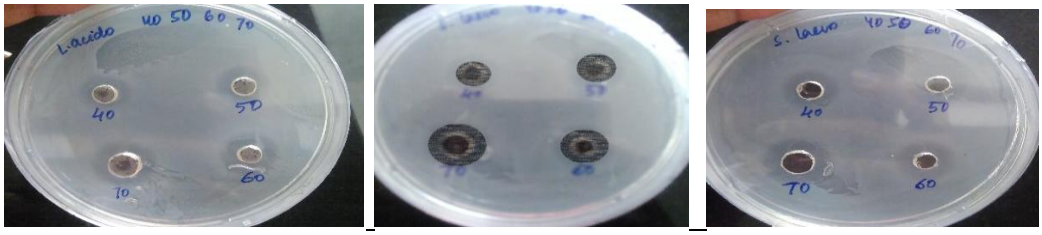
Zones of inhibition of *Bacillus cereus* were observed at 40, 50, 60, 70, 80, 90 and 100 µl of CFSs. However, 100 µl was found to be the optimum volume where a mild inhibitory activity was observed. CFSA, CFSB and CFSC showed the only weak inhibition against *B. cereus* with 0.9±0.01cm, 0.5±0.02cm and 0.1±0.01cm zones of inhibition. Figure 3 shows the ZOI on MHA agar plates.

Cell free supernatants of *L*.GG, *L. acidophilus* and *S. laevolacticus* showed moderate to strong inhibitory activity against the food borne pathogens *S. typhimurium* and *E. coli* O157:H7, however they showed weak inhibition against spore forming *B. cereus*. *L*.GG-CFS showed maximum inhibitory activity against all tested pathogens. The antimicrobial activity of the lactic acid bacteria may be due to a number of factors. Among these are decreased pH levels, competition for substrate, and the production of substances with bacteriocidal or bacteriostatic action including bacteriocin (Diez-Gonzalez, 2007). In fact, the drop in pH arising from the production of lactic is enough to inhibit certain strains. This is because the non-dissociated form of lactic acid triggers a lowering of the internal pH of the cell that causes a collapse in the electrochemical proton gradient in sensitive bacteria, hence a bacteriostatic or bacteriocidal effect (Parente and Ricciardi 1999). Inhibitory activity of *Lactobacillus CFS* against various food-borne pathogens has been reported previously (Arena *et al.,* 2016; Sharma *et al.,* 2017).

Table.1 Inhibitory activity of LGG-CFS at various conditions

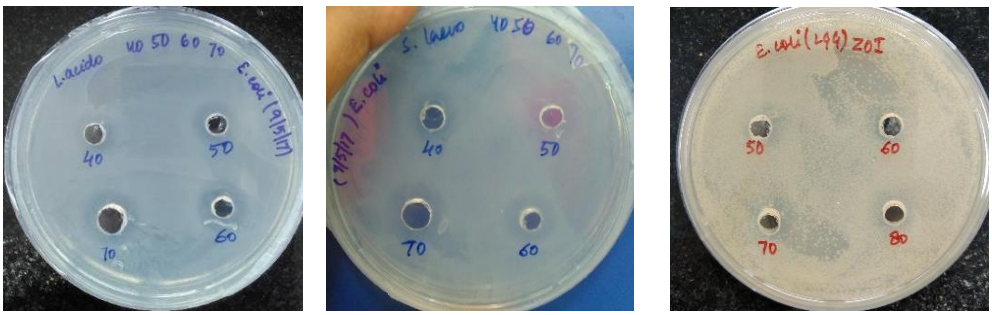
Condition	Inhibitory Activity CFSA	Inhibitory Activity CFSB	Inhibitory Activity CFSC
pH3	+	+	+
pH4	+	+	+
pH5	+	+	+
pH6	-	-	-
pH8	-	-	-
pH10	-	-	-
Papain	-	-	-
Lyophilization	+	+	+
60 °C	+	+	+
80°C	+	+	+
Autoclaving	+	+	+

Fig.1 Zone of inhibition showing inhibitory activity of CFS towards *S. Typhimurium*



L.acidophilus-CFS LGG-CFS L.laevolacticus-CFS

Fig.2 Zone of inhibition showing inhibitory activity of CFS towards *E. coli*



L.acidophilus-CFS L.laevolacticus- CFS L.GG- CFS

Fig.3 Zone of inhibition showing inhibitory activity of CFS towards *Bacillus*

L.acidophilus-CFS **L.laevolacticus- CFS** **L.GG- CFS**

The key factor for commercialization of the any product is its temperature stability which basically tells the shell-life of that product. Any product which can withstand the temperature fluctuations shows longer shelf-life as compared to the product which is temperature liable. Inhibitory molecule present in CFSs of tested *Lactobacillus* strains well tolerated the higher temperature even autoclaving. As it also tolerated the process of lyophilization, CFSs can be dried for commercial preparations. The inhibitory metabolites showed activity at acidic pH over pH range of 3-5 however lost their activities at pH 6 and alkaline pH 8 and 10 (Table 1). CFSA, CFSB and CFSC lost their activities after treatment with papain enzyme.

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