IN VITRO STUDY ANALYSIS OF ANTIMICROBIAL PROPERTIES OF LACTIC ACID BACTERIA AGAINST PATHOGENS

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ABSTRACT

In recent years, interest in Lactic Acid Bacteria (LAB) has grown considerably due to their potential fermentation activity in the production of foods and for the vast array of health benefits they confer. The viability of probiotic bacteria is an essential factor for human immunity. This depends on the ability of the bacteria to survive adverse conditions in the gastrointestinal tract. A well-known attribute of LAB is its ability to produce certain antimicrobial metabolites such as organic acids (lactic acid and acetic acid), hydrogen peroxide and bacteriocins that are known to inhibit pathogenic bacteria and prevent their colonizing in the gut. Therefore an attempt was made to study and elucidate the antimicrobial activity of *Lactobacillus bulgaricus, Lactobacillus casei* and *Lactobacillus bulgaricus* with *Streptococcus thermophilus*. Results indicate mix culture of *L.bulgaricus* and *S. thermophilus* had the highest antimicrobial activity against *S. aureus. L. bulgaricus* had high antimicrobial activity against *S. aureus* and lowest against *E.coli*. Similar antimicrobial efficiency was seen in *L.casei*. These results suggest that LAB could be useful as a probiotic to increase immunity of a host through dietary supplementation.

Key words: *L. bulgaricus, S. thermophilus, L. casei, S. aureus, E. coli,* Probiotics, Pathogens Lactic acid bacteria (LAB), antimicrobial property

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INTRODUCTION

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria that produce lactic acid as their main fermentation product (Mathur, 2005). Typical LAB members are Gram-positive, facultative anaerobic, catalase negative organisms with low G+C content. Most LAB have a long history of being consumed as part of traditional fermented foods and have been awarded the status of "Generally Regarded As Safe" (GRAS) by the Food and Drug Administration (FDA) (Ammor *et al*., 2007). The LAB group comprises the genera *Lactobacillus, Streptococcus, Lactococus, Leuconostoc, Pediococcus, Aerococcus, Alloicoccus, Dolosigranulum, Enterococcus, Globicatella, Lactospaera, Oenococcus, Carnobacterium, Tetragenococcus, Vagoccus* and *Weissella* (Ko and Ahn, 2000). Lactobacilli are naturally present or deliberately added as starter cultures in unpasteurized milk and dairy products such as cheeses, yogurts and fermented milks (Coeuret *et al.*, 2004).Yoghurt is a common product in which probiotic bacteria can be delivered to the human lower gut is made by fermentation of milk with the starter cultures *Lactobacillus delbrueckii* subspecies *bulgaricus* and *Streptococcus thermophilus* (Hamilton-Miller, 2004).LAB produces various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations. The bacteriocins from the lactic acid bacterial isolates generally recognized as safe (GRAS). Bacteriocins are antimicrobial proteinaceous compounds that are inhibitory towards sensitive strains and are produced by both Gram-positive and Gram-negative bacteria (Padmanabha *et al.*, 2006). Bacteriocins are protein or peptides, which do not harm the producer strain but have lethal antibacterial activity against food spoilers and/or food borne pathogens (Rodriguez *et al*., 1989). Most of the bacteriocins from LAB have been isolated from species of the genus *Lactobacillus* (Klein *et al*., 1998). Different antimicrobials such as lactic acid, acetic acid, hydrogen peroxide, carbon dioxide and bacteriocins produced by these bacteria can inhibit pathogenic and spoilage microorganisms extending the shelf-life and enhancing the safety of food products (Grobben *et al*., 1998)LAB offers the host protection against disease, and promotes normal intestinal function (Fooks and Gibson, 2002). These microorganisms survive the passage through the gastrointestinal tract and eventually establish in the colon. However, they must be taken regularly and at sufficiently high levels to avoid washout and to ensure sustained benefits (Peres *et al.*, 2012). Their benefits are related to the prevention of growth of harmful bacteria by competitive exclusion and by the production of organic compounds. Associated effects of probiotics include prevention and treatment of diarrhoea, alleviation of lactose intolerance, immune modulation and prevention or alleviation of allergies in children (Erkkilä and Petäjä, 2000). Lactobacilli have been demonstrated to have numerous potentially important benefits in terms of gut health and immunity. They can stimulate immune mechanisms at the intestinal level, increase immunoglobulin secretion, enhance antigen presentation and macrophage activation, and inhibit mucosal attachment of pathogens. (Tanriover *et al.*, 2012). Their benefits are related to the prevention of growth of harmful bacteria by competitive exclusion and by the production of organic compounds. Associated effects

of probiotics include prevention and treatment of diarrhoea, alleviation of lactose intolerance, immune modulation and prevention or alleviation of allergies in children (Erkkilä, and Petäjä, 2000). Translocation of viable or probiotic bacteria in minute amounts constitutes a physiologically important boost to the immune system (Lichtman, 2001). Immunomodulatory and immunostimulatory functions (Fooks and Gibson, 2002). Management of inflammatory bowel diseases (Gill and Guarner, 2004), treatment of infections during pregnancy, management of allergic diseases, control of antibiotic-related diarrhoea and prevention of urinary tract infections, amongst others (Jayne *et al.,* 2014), alleviation of lactose intolerance (Marteau *et al*., 1990). It is speculated that inflammation associated with rheumatoid-arthritis may be modulated by the use of probiotics (Marteau *et al.,* 2001). The beneficial effects of probiotics depend on their colonization of the gut and their effect on harmful bacteria, for which certain functional properties are necessary (Hyronimus *et al.,* 2000). They are capable of adhering to human epithelial cells; they prevent colonization by pathogenic bacteria, either by immune exclusion, competitive adhesion or synthesis of antimicrobial substances (Casula and Cutting, 2002; Hyronimus *et al*., 1998).

As stated the pros of probiotics depends on the efficiency to tolerate and colonize within the gastrointestinal tract. Therefore the main aim of the current study was focused on the antimicrobial activity of Lactobacilli.

MATERIALS AND METHODS

CULTURE MAINTENANCE

L. bulgaricus and *S. thermophilus* were procured from National Dairy Research Institute, Bengaluru. It was maintained in skim milk, incubated at 42˚C for 4 hours for *S. thermophilus* and 6 hours for *L. bulgaricus* and then refrigerated at 4˚C. Subculturing was done every 7 days. *L. casei,* isolated from Yakult was maintained in skim milk, incubated at 37˚C for 5 hours and then refrigerated at 4˚C. Subculturing was done every 7 days.

Escherichia coli was isolated from sewage using Eosin Methylene Blue agar (EMB agar) by spread plate method. Colonies with green metallic sheen were selected and sub cultured in Nutrient agar at 37˚C for 24 hours and then refrigerated at 4˚C. Subculturing was done every 7 days. *Staphylococcus aureus* was procured from Microbial Type Culture Collection, Chandigarh (MTCC number 3160) and was sub cultured in Nutrient agar at 37˚C for 24 hours and then refrigerated at 4˚C. Subculturing was done every 7 days.

ANALYSIS OF ANTIMICROBIAL ACTIVITY OF LAB

Preparation of cell free extract

The selected LAB species were inoculated to 50ml MRS broth and incubated at 37˚C for 4 and 18hrs. It was centrifuged separately at 10,000 × g for 15 minutes. The supernatant was collected and passed through 0.20 µm sterile syringe filter. The cell free supernatant broth was collected for the

antibacterial study against selected pathogens (Astha *et al*., 2012).

Antibacterial activity test by agar well diffusion method

The agar well diffusion method was used to determine the antibacterial property of the LAB. A 24 hr culture of the pathogens (*E. coli* and *S. aureus*), grown in nutrient broth at 37˚C was used. A lawn of the indicator strain was swabbed on nutrient agar plates. The plates were allowed to dry and a sterile cork borer of diameter (5mm) was used to cut uniform wells in the agar. Each well was filled with 60 µL culture free filtrate and cell suspended in MRS broth obtained from the LAB isolates after 4 hrs and 18 hrs incubation. After incubation at 37˚C for 24 hrs, the plates were observed for a zone of inhibition (ZOI) around the well (Astha *et al*., 2012). T – test was carried out to determine the significance of variation in anti- microbial activity between cell suspension and cell free extract.

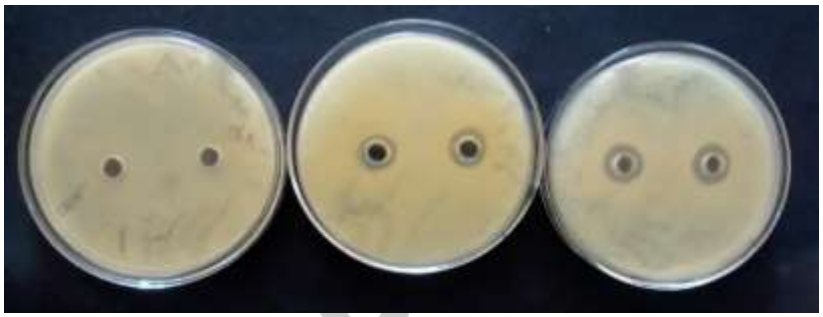
RESULTS AND DISCUSSION

An essential condition for LAB with probiotic activity is the productive capacity of inhibitory substances that antagonize pathogenic strains (Nemcova, 1997). The antimicrobial effect exerted by LAB is due to the production of lactic acid and reduction of pH, and acetic acid, diacetyl, hydrogen peroxide, fatty acids, aldehydes, bacteriocin and other compounds (Daeschel, 1989; Jay, 1982).

The agar well diffusion method was used to assess the antibacterial activity of the selected LAB. Their antibacterial properties were tested against pathogenic bacteria namely *E. coli* and *S. aureus* (Tables 1 & 2, Figures 1, 2, 3, & 4). The antimicrobial activity was highest in the cell suspension in MRS after 18 hrs incubation. Results indicate that *S. thermophilus* with *L. bulgaricus* had the highest antibacterial property, was highest against *S. aureus* with a ZOI of 10.5±0.35 mm and for *E.coli*. 4±0mm. *L. bulgaricus* also showed antibacterial property against all tested pathogens with its activity being highest against *S. aureus* (10 ± 0 mm) and least against *E. coli* (3.5 ± 0.35 mm). *L. casei* also showed antibacterial property against all tested pathogens with its activity being highest against *S. aureus* (10 ± 0 mm) and least against *E.coli* (4.0 ± 0 mm). As a part of statistical analysis, the Student T-test showed there is a significant variation in anti- microbial activity between cell suspension and cell free extract except for *S. aureus* 4 hours incubation (*p≤ 0.05). Current finding is similar to the results reported by Gilliland and Speck (1977), that lactobacilli showed stronger antibacterial properties against Gram positive bacteria (*S. aureus* and *Clostridium perfringens*) than Gram negative bacteria (*E. coli* and *S. typhimurium*). Earlier reports (Tagg *et al*., 1976, Daeschel and Klaenhamner *et al*., 1985, Sanni *et al*., 1999) have shown that some bacteriocins produced by Gram-positive bacteria have a broad spectrum of activity.

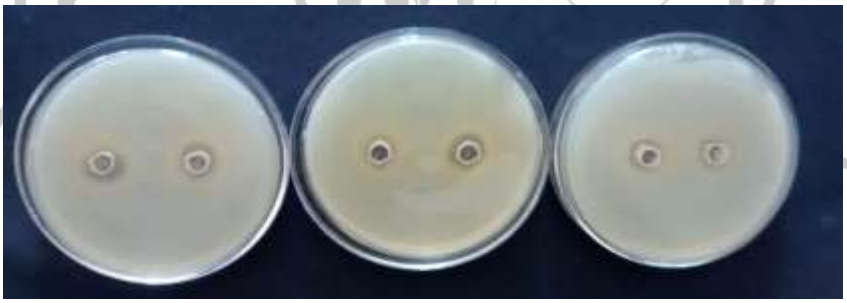
L. casei *L. bulgaricus* *S. thermophilus* and *L. bulgaricus*

Fig 1: Antimicrobial activity of a cell suspension against *S. aureus* after an 18hr incubation period.



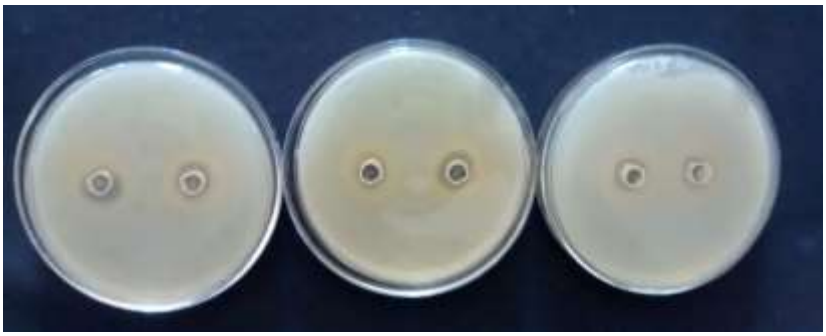
L. casei *L. bulgaricus* *S. thermophilus* and *L. bulgaricus*

Fig 2: Antimicrobial activity of a cell free extract against *S. aureus* after an 18hr incubation period.



L. casei *L. bulgaricus* *S. thermophilus* and *L. bulgaricus*

Fig 3: Antimicrobial activity of a cell suspension against *E. coli* after an 18hr incubation period.



L. casei *L. bulgaricus* *S. thermophilus* and *L. bulgaricus*

Fig 4: Antimicrobial activity of a cell free extract against *E.coli* after an 18hr incubation period.

Table 1: Antimicrobial activity of LAB after 4 hours incubation

Sample	Zone of Inhibition (in mm)			
	Cell suspended in MRS		Cell free extract	
	S. aureus	***E. coli***	***S. aureus***	***E. coli***
L. bulgaricus	8.5±0.35	4±0.707	7.5±1.06	1.5±0.35
S. thermophilus + *L. bulgaricus*	8.5±0.35	3.5±0.35	8±0.7	3±0.7
L. casei	8.5±0.35	3.5±0.35	7.5±1.06	2.5±0.35

Table 2: Antimicrobial activity of LAB after 18 hours incubation

SAMPLE	Zone of Inhibition (in mm)			
	Cell suspended in MRS		Cell free extract	
	S. aureus	***E. coli***	***S. aureus***	***E. coli***
L. bulgaricus	10±0	3.5±0.35	8±0	2±0
S. thermophilus + *L. bulgaricus*	10.5±0.35	4±0	10±0	3.5±0.35
L. casei	10±0	4±0	8.5±0.35	2.5±0.35

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REFERENCES

Mathur, S., and Singh, R. (2005). Antibiotic resistance in food lactic acid bacteria—a review. International Journal of Food Microbiology, 105(3), 281-295.

Ammor MS, Mayo B (2007) Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: an update. Meat Sci 76:138–146

Ko, S. H., and Ahn, C. (2000). Bacteriocin production by Lactococcus lactis

KCA2386 isolated from white kimchi. Food Science and Biotechnology, 9(4), 263-269.

Coeuret, V., Gueguen, M., & Vernoux, J. P. (2004). In vitro screening of potential probiotic activities of selected lactobacilli isolated from unpasteurized milk products for incorporation into soft cheese. Journal of dairy research, 71(04), 451-460.

Hamilton-Miller, J. M. T. (2004). Probiotics and prebiotics in the elderly.Postgraduate medical journal, 80(946), 447-451..

Padmanabha Reddy, V., Christopher, M. D., and Reddy, I. S. (2006). Antimicrobial activity of Lactobacillus acidophilus. Tamilnadu J Vet Animal Sci,2(4), 142-144.

Rodriguez, J. M., Sobrino, O. J., Fernandez, P. E., Hernandez, P. E., and Sanz, B. (1989). Antimicrobial activity of lactic acid bacteria isolated from Spanish dry fermented sausages. In 35. International Congress of Meat Science and Technology, Copenhagen (Denmark), 20-25 Aug 1989. SFI.

Klein, G., Pack, A., Bonaparte, C., and Reuter, G. (1998). Taxonomy and physiology of probiotic lactic acid bacteria. International journal of food microbiology, 41(2), 103-125.

Grobben, G. J., Chin-Joe, I., Kitzen, V. A., Boels, I. C., Boer, F., Sikkema, J. & De Bont, J. A. M. (1998). Enhancement of Exopolysaccharide Production by Lactobacillus delbrueckii subsp. bulgaricus NCFB 2772 with a Simplified Defined Medium. Applied and environmental microbiology, 64(4), 1333-1337.

Fooks L J and Gibson GR (2002). Probiotics as modulators of the gut flora. Br J Nutr, 88, 39-49.

Erkkilä, S., Venäläinen, M., Hielm, S., Petäjä, E., Puolanne, E., & Mattila-Sandholm, T. (2000). Survival of Escherichia coli O157: H7 in dry sausage fermented by probiotic lactic acid bacteria. Journal of the Science of Food and Agriculture, 80(14), 2101-2104.

Tanriover, M. D., Aksoy, D. Y., & Unal, S. (2012). Use of probiotics in various diseases: evidence and promises. Pol Arch Med Wewn, 122(suppl 1), 72-77.

Lichtman, S. M. (2001). Baterial translocation in humans. Journal of pediatric gastroenterology and nutrition, 33(1), 1-10.

Gill, H. S., & Guarner, F. (2004). Probiotics and human health: a clinical perspective. Postgraduate Medical Journal, 80(947), 516-526.

Hyronimus, B., Le Marrec, C., Sassi, A. H., & Deschamps, A. (2000). Acid and bile tolerance of spore-forming lactic acid bacteria. International journal of food microbiology, 61(2), 193-197.

Casula, G., & Cutting, S. M. (2002). Bacillus probiotics: spore germination in the gastrointestinal tract. Applied and environmental microbiology, 68(5), 2344-2352.

Hyronimus, B., Le Marrec, C., & Urdaci, M. C. (1998). Coagulin, a bacteriocin-like-inhibitory substance produced by Bacillus coagulans I. Journal of applied microbiology, 85(1), 42-50.

Marteau, P. R., de Vrese, M., Cellier, C. J., and Schrezenmeir, J. (2001). Protection from gastrointestinal diseases with the use of probiotics. The American journal of clinical nutrition, 73(2), 430s-436s.

Marteau, P., Flourie, B., Pochart, F., Chastang, C., Desjeux, J.F. and Rambaud, J.C. (1990) Effect of the microbial lactase

(EC 3.2.1.23) activity in yoghurt on the intestinal absorption of lactose: an in vivo study in lactase-deficient humans. Br J Nutr 64, 71–79.

Nemcova, R. 1997. Criteria for selection of lactobacilli for probiotic use. Vet. Med. 42: 19-27

Daeschel MA. (1989). Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technology 43: 164-166.

Jay JM. (1982). Antimicrobial properties of diacetyl. Applied and Environmental Microbiology 44: 525-532.

Gilliland, S., Speck, M. (1977). Antagonistic action of Lactobacillus acidophilus towards intestinal and foodborne pathogens in associative cultures J. Food Protect, 40:820-823.

Tagg J. R, A. S. Dajani, and L. W. Wannamaker (1976). Bacteriocins of gram positive bacteria, Bacteriol. Rev., vol. 40, pp. 722-756.

Daeschel M. A and T. R. Klaenhamner (1985). Association of a 13.6 megadalton plasmid in Pediococcus pentosaceus with bacteriocins acidity, Appl. Environ. Microbiol., vol. 50, pp. 1538-1541.

Sanni A I, A. A. Onilude, S. T. Ogunbanwo, and S. I. Smith (1999). Antagonistic activity of bacteriocin produced by Lactobacillus species from Ogi, an indigenous fermented food, J. Basic Microbiol., vol. 39, pp. 189-195.

Erdourul Z. and F. Erbulur (2006). Isolation and Characterization of Lactobacillus bulgaricus and Lactobacillus casei from various foods, Turk. J. Biol., vol. 30. pp. 39-44.

Akpinar A, O. Yerlikaya, and S. Kiliç (2011). Antimicrobial activity and antibiotic resistance of Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus strains isolated from Turkish homemade yoghurts, Afr. J. Microbiol. Res., vol. 5(6), pp. 675-682.

Erkus O (2007), Isolation, phenotypic and genotypic characterization of yoghurt starter bacteria, Master Thesis, The Graduate School of Engineering and Sciences of Izmir Institute of Technology, Turkey.

Fernandes C. F. and K. M. Shahani (1989). Modulation of antibiosis by lactobacilli and yogurt and its helpful and beneficial significance, Nutritional and Health Properties, pp. 145-160.

Kim H.S (1988). Characterization of Lactobacilli and Bifidobacteria as applied to dietary adjuvants, J. Cult. Dairy. Prod., vol. 23, pp. 6-9.

Gorbach S.L, T. W. Chang, and B. R. Goldin (1987).Successful treatment of relapsing Clostridium difficile colitis with Lactobacillus, GG. Lancet, vol. 2, pp. 1519, 1987