The Pharma Innovation Journal 2022; 11(12): 989-993

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(12): 989-993

www.thepharmajournal.com
Received: 07-09-2022
Accepted: 09-10-2022

Pallavi Shelar
Department of Dairy Microbiology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Sarang Dilip Pophaly
Department of Dairy Microbiology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Manorama
Department of Dairy Microbiology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Awadhesh Kumar Tripathi
Department of Dairy Microbiology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Shakeel Asgar
Department of Dairy Technology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Archana Khare
Department of Dairy Chemistry, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Lata Jain
National Institute of Biotic Stress Management (ICAR-NIBSM), Baronda, Raipur, Chhattisgarh, India

Virendra Kumar
Department of Dairy Microbiology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Corresponding Author:
Pallavi Shelar
Department of Dairy Microbiology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Development of Post biotic preparation using *Lactococcus* isolates

Pallavi Shelar, Sarang Dilip Pophaly, Manorama, Awadhesh Kumar Tripathi, Shakeel Asgar, Archana Khare, Lata Jain and Virendra Kumar

Abstract
Postbiotics are the soluble factors either synthesized by cells or part of cellular structure which have certain physiological benefits to the host. In the present study, a previously isolated strain *Lactococcus lactis* sub sp. *lactis* KD2 showing high antimicrobial activity, was used to prepare two different types of postbiotic formats, heat killed *Lactococcus* (HKL) and cell free supernatant (CFS)**.** In both the cases the cells were heat killed at tested temperatures in presence of induced osmotic stress by sucrose. The optimum combination of heat and sucrose was then used for preparing both the postbiotic formats. The two postbiotic preparations were then tested for antimicrobial activity against selected pathogens and fungi.

Keywords: *lactococccus*, postbiotics, heat killed cells, cell free supernatant, antagonistic activity

Introduction
The assemblage of microorganisms that inhabit the human body, their genomes and metabolites, as well as the environment in which they live, is called the microbiota. The past few decades have demonstrated unequivocally the importance of the human microbiota to both short-term and long-term human health.
The definition given by the FAO/WHO (2001) for probioitcs are live microorganisms which when administered in adequate amount confer a health benefit on the host [1, 3, 8], whereas a prebiotic is a "substrate that is selectively utilized by host microorganisms conferring a health benefit" [7]. A synbiotic, initially conceived as a combination of both probiotics and prebiotics, has now been defined as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" [21]. The concept of postbiotics is related to this family of terms and is emerging as an important microorganism-derived tool to promote health. The term "postbiotics" was chosen by the panel as a composite of 'biotic', defined as "resulting to or relating from living organisms and 'post', a prefix meaning 'after'. Together these terms suggest 'after life'; that is, non-living organisms. Other related terms have also been used, including 'paraprobiotics' [6, 11, 13, 20], 'ghost probiotics' [22], 'tyndallized probiotics' [12, 15], 'parapsychobiotics' [14], 'metabiotics' [17, 19] and 'bacterial lysates' [10]. In addition the term "postbiotics" has been proposed to define "preparation of inanimate microorganisms and/or their components that confer a health benefit on the host". By contrast, postbiotics are the complex mixture of healthy metabolic products or secreted components of probitotics in cell-free supernatants such as enzymes, secreted proteins, short chain fatty acids, vitamins, amino acids, peptides, organic acids etc.
Even though the classical definition of probiotics indicate that they should be alive in order to provide health benefits to the hosts, recent studies have proved that inactivated probiotic microorganisms (referred as postbiotics) can also provide such as benefits [5, 9, 16, 23].
LAB are gram positive, facultative anaerobic, catalase negative and motile. They belong to the order *Eubacteriales* under *Streptococcaceae* and *Lactobacillaceae* families. The LAB plays important roles in ensuring the protection and safety of dairy products through production of antimicrobial agents including lactic acid, diacetyl, hydrogen peroxide, and bacteriocins. Bacteriocins, which have the potential to serve as food preservatives, have been identified in dairy starter cultures. Strains of the genus *Lactococcus* have cocci morphologies and form single cocci pairs or short chain formations (0.5–1.5 μm) in their growth medium. Their optimum growth temperature is 30 °C and they can grow between 10 °C and 45°C. *Lactococcus lactis* can mostly be isolated from dairy products including raw milk and kefir grains.

It is also used in starter cultures used for production of different dairy products including cheese. Based on its history of use in food fermentations, *L. lactis* has GRAS (generally regarded as safe) status. *Lactococcus lactis* produces lactic acid in dairy starter cultures and help to break down milk proteins during fermentation, contributing to the sensory and microbiological properties of the product [4]. In this study, the aim of this study was to determine the development of postbiotic preparation using *Lactococcus* isolate (KD2) and antimicrobial activities of heat killed *Lactococcus* and cell free supernatant produced from whey based medium.

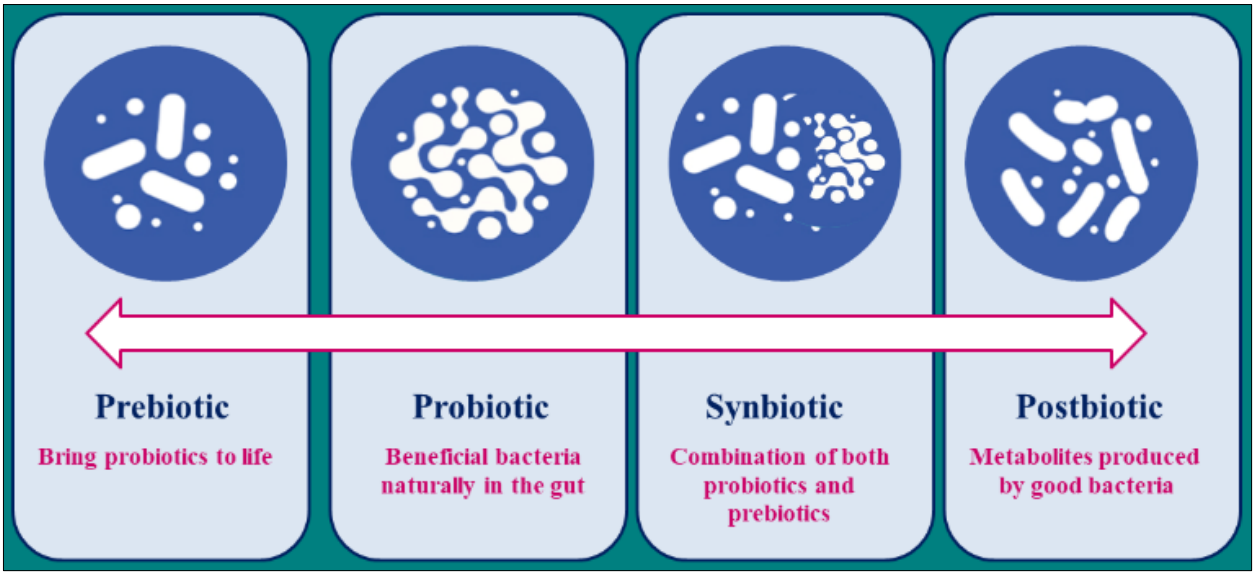

Fig 1: Definitions of the terms probiotics, prebiotics, synbiotics and postbiotics.

2. Material and Methods

2.1 Preparation of Whey Based Medium

A whey based medium for growth of *L. lactis* was prepared [18]. Sweet whey powder was procured from Parag Milk Foods Limited and stored at refrigeration temperature. Whey Powder was weighed in required quantity and reconstituted in distilled water to obtain 6% total solid. The pH of the solution was adjusted to 4.0 using 6N HCl and then autoclaved at 121°C for 15 min. which resulted in precipitation of excess whey proteins. The supernatant portion was carefully decanted and collected in pre-sterilized flasks. Then autoclaved solution of yeast extract (12g/L), tryptone (5g/L) was added to prepare the whey based medium. The pH of the medium was adjusted to 7.0 using 6N autoclaved NaOH.

2.2 Preparation of Postbiotics (Heat killed postbiotics and Cell free postbiotics)

2.2.1 Heat and Sugar Combination

The postbiotic preparation was analysed for no viability after heating at varying temperature-time combination and sugar concentration.

Table 1: Different combinations of heating and sucrose concentration used for prepration of postbiotic

Heat (Temperature/Time)	Sucrose
60 °C/ 10 min	0 per cent
	4 per cent
	5 per cent
	6 per cent
70 °C/ 10 min	0 per cent
	4 per cent
	5 per cent
	6 per cent
80 °C/ 10 min	0 per cent
	4 per cent
	5 per cent
	6 per cent
90 °C/ 10 min	0 per cent
	4 per cent
	5 per cent
	6 per cent
100 °C/ 10 min	0 per cent
	4 per cent
	5 per cent
	6 per cent

2.2.2 Preparation of Heat Killed *Lactococcus* (HKL)

Lactococcus (KD2 isolates) was grown in whey based medium at 30 °C for 24-48 h. To obtain live lactococci suspension, the cells were then harvested by centrifugation (10,000 rpm/ 10 min/ 4 °C) and washed using sterilized saline. The supernatant (spent whey based medium broth) was discarded. The cell pellet was washed twice using sterilized saline. Cell suspension of 10^8 cells/ml was standardized in a saline solution using a spectrophotometer (650 nm). The suspension containing 10^8 cells/ml was heated at different time/temperature and sucrose combination (as mentioned in 2.3.1) to prepare Heat Killed *Lactococcus* (HKL).The sterility of HKL suspension was tested by plating one ml of the suspension on M-17 broth and confirmed by absence of colonies on the plate after 24 hrs.

2.2.3 Preparation of Cell Free Supernatant (CFS)

For preparation of cell free postbiotic, a cell free supernatant of the culture was prepared and heat treated. Whey based Medium for growth of lactococci (KD2 isolates) was prepared as mentioned in (2.2) Fifty ml of whey based medium was inoculated with 1 ml of culture. The cell number was standardized to 10^8 cells/ml by adjusting the OD (650 nm) using a spectrophotometer. The cells were then centrifuged at 10,000 rpm/ 10 min/ 4°C and cell pellet was discarded. The supernatant was then heated at different time/temperature and sucrose combination (as mentioned in 2.3.1) to kill any residual cells remained after centrifugation. The sterility of Cell Free Suspension (CFS) was tested by plating one ml of the suspension on M-17 broth and confirmed by absence of colonies on the plate after 24 hrs.

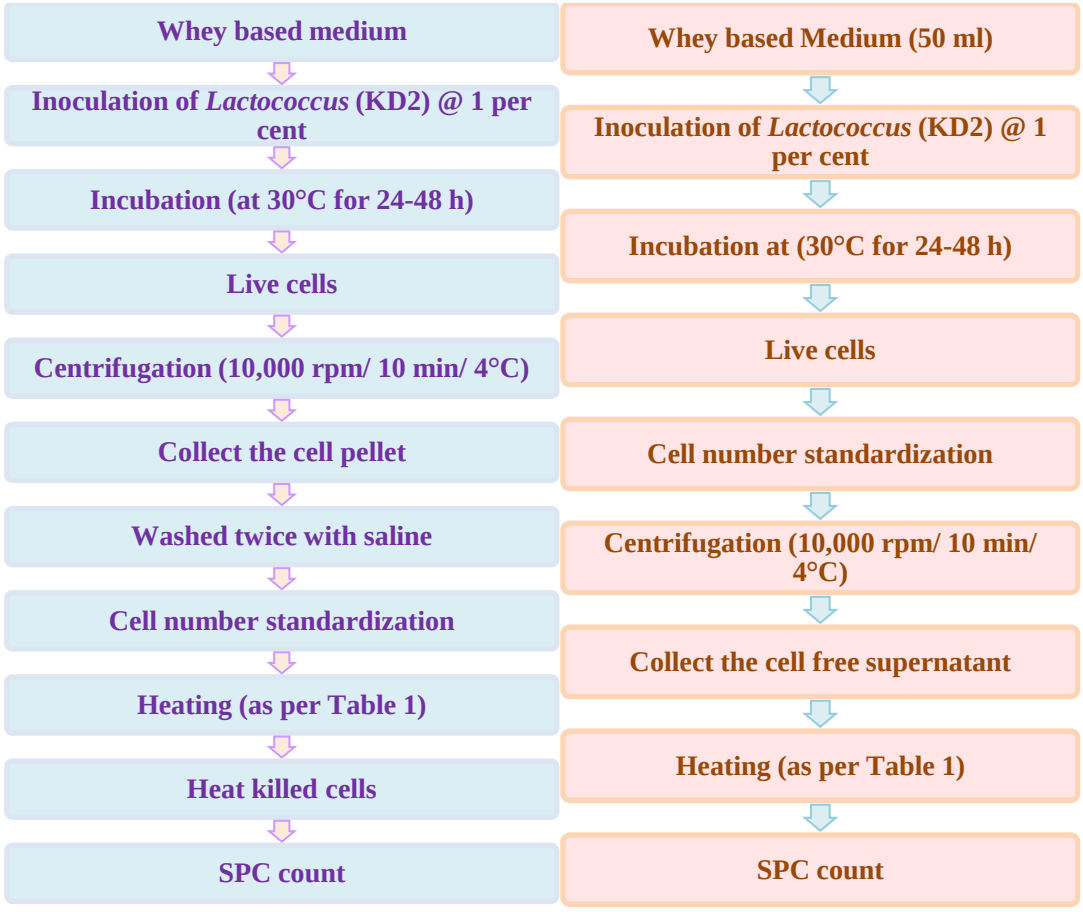

Fig 2: An outline on preparation process for the heat killed *Lactococcus* [A] and cell free supernatant [B] from whey based medium

3. Result and Discussion

3.1 Heat and Sucrose Combination

For preparation of postbiotics, different combination of heat and sucrose were used as described in table 2. The combinations were then tested for viability by standard plate count. In general, a combination of sucrose and heating induced more drastic killing of cells in comparison to only heating.

Table 2: The preparation of HKL and CFS was treated by heat and sucrose combinations and number of viable cells.

Heat (Temperature/Time)	Sucrose	SPC Count
60 °C/ 10 min	0 per cent	$5.6x10^5$ cfu/mL
	4 per cent	$5x10^5$ cfu/mL
	5 per cent	$5.5x10^4$ cfu/mL
	6 per cent	$5.8x10^4$ cfu/mL
70 °C / 10 min	0 per cent	$5.6x10^6$ cfu/mL
	4 per cent	$5.8x10^6$ cfu/mL
	5 per cent	$5.9x10^5$ cfu/mL

	6 per cent	$4x10^3$ cfu/mL
80 °C/ 10 min	0 per cent	$5.7x10^4$ cfu/mL
	4 per cent	$5.8x10^3$ cfu/mL
	5 per cent	$5x10^3$ cfu/mL
	6 per cent	$5.2x10^2$ cfu/mL
90 °C/ 10 min	0 per cent	$5.1x10^2$ cfu/mL
	4 per cent	48 cfu/mL
	5 per cent	Nil
	6 per cent	Nil
100 °C / 10 min	0 per cent	Nil
	4 per cent	Nil
	5 per cent	Nil
	6 per cent	Nil

3.2 Antagonistic effects of heat killed lactococci and cell free supernatant against indicator microorganisms.

The agar well diffusion assay was performed to detect antibacterial activities [2] of heat killed *Lactococcus* (HKL) and cell free supernatant (CFS) produced from whey based medium. The suspension of HKL was adjusted to McFarland standard and inoculated onto whey based medium obtained by centrifugation (10000 rpm/ 10 min/ 4°C). The plates were poured with 20 ml of brain heart infusion (BHI) agar. The indicator bacterial strains i.e., *E. coli*, *Bacillus cereus*, *Enterococcus faecalis* and *Staphylococcus aureus* was spread on the surface of BHI agar. Wells of 6 mm in diameter were cut into these agar plates and 100 µl of the HKL and CFS were placed into each well. The culture plates were incubated at 30 °C for 24-48 h and the zone of inhibition measured in diameter (mm). Data of antibacterial activities against bacterial pathogens were recorded according to the inhibition zone around the punched well. Strains with inhibition zones less than 5 mm in diameter, between 5 and 10 mm and more than 10 mm were classified as non-inhibitors, intermediate inhibitors and strong inhibitors, respectively. The assay was performed in duplicate and the inhibition zone of HKL was compared with that of CFS was recorded (Table 3). For the antimicrobial assay the following fungal strains used in this study were *Aspergillus*, *Penicillum*, *Mucor* and *Rhizopus*. Fungi were cultured on Potato Dextrose Agar plates for 25 °C for 5 days.

Table 3: Antimicrobial activity of HKL and CFS against the indicator microbial cultures

Indicator Microorganisms	Inhibition Zone Diameter (mm)	
	Heat killed *Lactococcus*	Cell free supernatant
E. coli	+++	++
Bacillus cereus	+++	-
Enterococcus faecalis	+++	+
Staphylococcus aureus	+++	+
Aspergillus	++	-
Penicillum	++	-
Mucor	+	-
Rhizopus	++	-

The following scale was used: (-) no inhibition zone, (+) inhibition zone =<5mm, (++) inhibition zone = 5-10 mm, (+++) inhibition zone = more than 10 mm.

Conclusion

Postbiotics are the milieu of compounds either synthesized by cells or part of cellular structure which have certain physiological benefits to the host. In the present study a previously isolated strain *Lactococcus lactis* subsp. *lactis* KD2 showing high antimicrobial activity, was used to prepare two different types of postbiotic formats, heat killed *Lactococcus* (HKL) and Cell free supernatant (CFS). A combination of heat and varying sucrose concentration was tested to find the optimum conditions for both types of postbiotic preparation which has no viable cells present in the system. A combination of 5% sucrose and a temperature time combination of 90 °C/10 min resulted in zero viability after analysis. All the combinations tested above this limit also resulted in similar results. After standardization of postbiotic preparation, both the types were tested for antimicrobial activity against common pathogens and fungi. Heat killed *Lactococcus* (HKL) showed a considerably high antimicrobial activity against all the tested pathogens and fungi in comparison to cell free supernatant. The antimicrobial activity can be attributed to the intracellular compounds synthesized by the culture. The inanimate postbiotic preparation prepared from the culture after heat killing of cells, retained the antimicrobial properties.

Acknowledgement

The authors would like to express their gratitude towards the Vice-chancellor of Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Durg for providing all the essential support for this study.

References

1. Adams CA. The probiotic paradox: Live and dead cells are biological response modifiers. Nutrition Research Reviews. 2010;23:37-46.
2. Amin M, Adams M, Bolch CJ, Burke CM. *In vitro* screening of lactic acid bacteria isolated from gastrointestinal tract of Atlantic salmon (*Salmo salar*) as probiont candidates. Aquaculture International. 2017;25(1):485-498.
3. Ananta E, Knorr D. Comparison of inactivation pathways of thermal or high pressure inactivated *Lactobacillus rhamnosus* ATCC 53103 by flow cytometry analysis. Food Microbiology. 2009;26:542-546.
4. Cavanagh D, Fitzgerald GF, McAuliffe O. From field to fermentation: the origins of *Lactococcus lactis* and its domestication to the dairy environment. Food microbiology. 2015;47:45-61.
5. De Almada CN, Almada CN, Martinez RC, Sant'Ana AS. Paraprobiotics: Evidences on their ability to modify biological responses, inactivation methods and perspectives on their application in foods. Trends in Food Science & Technology. 2016;58:96-114.
6. Deshpande G, Athalye-Jape G, Patole S. Paraprobiotics for preterm neonates–the next frontier. Nutrients.

2018;10:871.
7. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, *et al*. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nature Reviews Gastroenterology & Hepatology. 2017;14(8):491-502.
8. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, *et al*. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology & Hepatology. 2014;11:506-514.
9. Imaoka A, Shima T, Kato K, Mizuno S, Uehara T, Matsumoto S. Anti-inflammatory activity of probiotic Bifidobacterium: Enhancement of IL-10 production in peripheral blood mononuclear cells from ulcerative colitis patients and inhibition of IL-8 secretion in HT-29 cells. World Journal of Gastroenterology. 2008;14:2511-2516.
10. Jurkiewicz D, Zielnik-Jurkiewicz B. Bacterial lysates in the prevention of respiratory tract infections. Otolaryngol. Pol. 2018;72:1-8.
11. Kanauchi O, Andoh A, AbuBakar S, Yamamoto N. Probiotics and paraprobiotics in viral infection: Clinical application and effects on the innate and acquired immune systems. Current Pharmaceutical Design. 2018;24:710-717.
12. Lopetuso L, Graziani C, Guarino A, Lamborghini A, Masi S, Stanghellini V. Gelatin tannate and tyndallized probiotics: a novel approach for treatment of diarrhea. European Review for Medical and Pharmacological Sciences. 2017;21(4):873-883.
13. Murata M, Kondo J, Iwabuchi N, Takahashi S, Yamauchi K, Abe F, *et al*. Effects of paraprobiotic *Lactobacillus paracasei* MCC1849 supplementation on symptoms of the common cold and mood states in healthy adults. Beneficial Microbes. 2018;9(6):855-864.
14. Nishida K, Sawada D, Kawai T, Kuwano Y, Fujiwara S, Rokutan K. Para- psychobiotic *Lactobacillus gasseri* CP 2305 ameliorates stress- related symptoms and sleep quality. Journal of Applied Microbiology. 2017;123(6):1561-1570.
15. Pique N, Berlanga M, Minana-Galbis D. Health benefits of heat- killed (tyndallized) probiotics: an overview. International Journal of Molecular Sciences. 2019;20:2534.
16. Rampengan NH, Manoppo J, Warouw SM. Comparison of efficacies between live and killed probiotics in children with lactose malabsorption. Southeast Asian Journal of Tropical Medicine and Public Health. 2010;41:474-481.
17. Sharma M, Chandel D, Shukla G. Antigenotoxicity and cytotoxic potentials of metabiotics extracted from isolated probiotic, *Lactobacillus rhamnosus* MD 14 on Caco-2 and HT-29 human colon cancer cells. Nutrition and Cancer. 2020;72:110-119.
18. Shekhar B. Production of *Lactococcus lactis* biomass through induced aerobic respiration in whey based medium. M.Tech. (Dairy Microbiology) Thesis, C.G.K.V. Raipur, Chhattisgarh; c2017.
19. Shenderov BA. Metabiotics: Novel idea or natural development of probiotic conception. Microbial Ecology in Health and Disease. 2013;24:20399.
20. Sugawara T, Sawada D, Ishida Y, Aihara K, Aoki Y, Takehara I, *et al*. Regulatory effect of paraprobiotic *Lactobacillus gasseri* CP2305 on gut environment and function. Microbial Ecology in Health and Disease. 2016;27(1):30259.
21. Swanson KS, Gibson GR, Hutkins R, Reimer RA, Reid G, Verbeke K, *et al*. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. Nature Reviews Gastroenterology & Hepatology. 2020;17(11):687-701.
22. Taverniti V, Guglielmetti S. The immunomodulatory properties of probiotic microorganisms beyond their viability (Ghost probiotics: Proposal of paraprobiotic concept). Genes & Nutrition. 2011;6:261-274.
23. Villena J, Barbieri N, Salva S, Herrera M, Alvarez S. Enhanced immune response to pneumococcal infection in malnourished mice nasally treated with heat-killed *Lactobacillus casei*. Microbiology and Immunology. 2009;53:636-646.