Enterocins: Symptomatic for Bio-alternative in Caries Control

Ramamoorthi Arularasi Aberna¹*, Kesani Prabhakar²

¹Lecturer, Department of Microbiology, Rajah Muthiah Dental College and Hospital, Annamalai University, Annamalainagar, India
²Professor, Department of Microbiology, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar, India

*Address for Correspondence: Dr. R. Arularasi Aberna, Lecturer, Department of Microbiology, Rajah Muthiah Dental College and Hospital, Annamalai University, Chidambaram-608002, India

ABSTRACT

Background- Enterocins are antimicrobial peptides produced by Enterococcus species, which have inhibitory activity on closely related genera. Streptococcus mutans has been implicated as a pivotal organism to initiate dental caries ensuing, serious impairment to structure and functions of tooth beside the mental health of humans. Hence we investigated the possibility of using enterocins in caries treatment and prophylaxis.

Methods- S. mutans was isolated from the saliva of 50 caries-prone humans. Enterococcus faecalis were isolated from the stool samples of humans. The species identity of the isolated organisms was confirmed using conventional biochemical methods. The inhibitory activity of enterocins on S. mutans isolated and their active concentrations was identified by spot-on-lawn assay. Inhibitory activity of 3 enterocins on their target S. mutans isolates were further analyzed by time-kill assay and colony forming units (CFU/ml) over 0, 4, 8 and 12 h time interval was determined.

Results- Enterocins produced by three E. faecalis isolates demonstrated inhibitory activity on more than 75% of S. mutans isolates. Enterocins SF101, enterocin SF118 and enterocin PF98 showed 100% inhibition of their target S. mutans at 1:2, 1:4, and undiluted concentrations. The viable count of enterocin treated S. mutans isolates declined to mean log₁₀ CFU/ml of 4.92 after 12 h time interval while the untreated control showed the increase to 9.11.

Conclusion- Enterocins exerts bactericidal activity against S. mutans, thus validating the possibility of enterocin to be used for caries treatment and prophylaxis.

Key-words: Dental caries, Enterocin, Inhibitory activity, Polymicrobial disease, Streptococcus mutans

INTRODUCTION

Dental caries is a polymicrobial disease resulting in damage to the crown and the root surface of the teeth [1]. Among the consortia of organisms causing caries, S. mutans plays a pivotal role due to its acidogenic, aciduric, biofilm forming and glucan-synthesizing ability [2]. There is an upsurge in the incidence of caries due to modifications in life-style and food habits.

In India, the disease is observed among 50% of children <5 years of age, 52.5% of children in 12 years, 61.4% among individuals aged 15, 79.2%, and 84.7% of population in the age group of 35–44 and 65–74 respectively [3]. Although the risk of serious detriment to systemic health is low among individuals with dental caries, it brings a whopping sequel in the quality of life of an individual by impairing physical appearance, tooth function, interpersonal relation and career opportunities [4]. Usage of pits and fissure sealant and fluoride agents are the mainstay for treating and preventing caries [5]. Enterococci are Gram positive cocci, facultative anaerobic organisms, belonging to the group of lactic acid bacteria (LAB). They inhabit human oral cavity, gastrointestinal tract and female genitalia. More than 28 species of Enterococci are reported however, E. faecalis is the commonly isolated species from humans [6,7]. This genus is reported to produce a class of bacteriocins (enterocins), which are small antimicrobial proteins or peptides. Enterocins exhibits inhibitory activity on other
Gram positive bacteria by bacteriostatic or bactericidal mode of action by creating pores on the cytoplasmic membrane and disrupting the osmotic stability of the cell [8,9]. The enterococogenic activity of Enterococcus from various sources has been identified and studied previously for its inhibitory action against microorganisms causing food putrefaction, biofilms, and pathogens [7,10].

The present study evaluated the inhibitory ability of enterocins produced by E. faecalis against S. mutans isolated from patients with dental caries. The study aims to elucidate the possibility of enterocins to be used as a bio-alternative in caries treatment and prophylaxis.

**MATERIALS AND METHODS**

**Isolation of E. faecalis** - Ten stool specimens submitted for bacteriological investigations to the Microbiology laboratory during August 2017 from patients attending the services of the Rajah Muthiah Institute of Health Sciences, Annamalai University, India was employed in the study. *E. faecalis* was isolated from the stool specimens by plating on Pfizer’s selective Enterococcus agar (Hi-Media Laboratories, India). Following aerobic incubation of the inoculated culture plates at 37°C, colonies morphologically resembling Enterococcus were characterized and identified as *E. faecalis* (test isolates) by standard microbiological and biochemical procedures [11]. These isolates were tested for their ability to produce enterocin, which would have inhibitory activity on S. mutans. All isolates were stored at -20°C in 20% glycerol Brain Heart Infusion broth (Sisco Research Laboratories Pvt. Ltd., India) until further use.

**Isolation of S. mutans** - The study population included 25 females and 25 males having a high incidence of dental caries and attending the services of the Rajah Muthiah Dental College and hospital, India. The study was incepted following institutional ethical committee clearance and written informed consent was obtained from patients. Un-stimulated saliva samples were collected from 50 patients belonging to the age group of 14–55 years. The samples were plated on Mitis Salivarius Bacitracin agar (Hi-Media Laboratories, India) containing 1% of potassium tellurite solution. Colonies resembling *S. mutans* were identified by standard microbiological and biochemical procedures [12]. These isolates were used to indicate (indicator isolates) the growth inhibitory effect of enterocins. The isolates were stored at -20°C in 20% glycerol Brain Heart Infusion broth (Sisco Research Laboratories Pvt. Ltd., India) until further use.

**Qualitative detection of Enterocin production** - The ability of the 10 *E. faecalis* isolates to produced enterocins, which would inhibited the *S. mutans* (indicator isolates) detected by agar spot assay described by Del Campo et al. [13] on bilayer trypticase soy agar plate. Plates were prepared with a bottom layer containing 1.5% agar and to the molten overlay agar containing 0.7% agar, 50 μl of each indicator isolate from overnight growth on Brain heart infusion broth was added, shaken to ensure even distribution and poured onto the surface of bottom agar layer and allowed to solidify. A single colony of test isolates from overnight growth on blood agar plates were spot inoculated into the indicator seeded bilayer trypticase soy agar plate using a sterile wooden pick at appropriately labeled sites. Inoculated plates were incubated for 48 h at 37°C in CO₂ environment. An isolate was considered as an enterocin producer when an inhibition zone of the indicator isolate was found around any of the test isolates.

**Quantitative detection of enterocin activity** - Three isolates of *E. faecalis*, which produced enterocin shown formidable activity as determined by the qualitative method was selected. The crude enterocin from these isolates was obtained as follows. *E. faecalis* was cultured in BHI broth and following an overnight incubation at 37°C, the culture was centrifuged twice at 15500x g for 15 min. The cell-free supernatant containing the enterocin was obtained by filtering the overnight culture through 0.45 μm pore size filter and the filtrate (crude enterocin) was stored at -20°C until use. Dilutions of the crude enterocin were prepared to range from 1:2 to 1:64 dilutions.

The various dilutions enterocins were tested for their inhibitory activity on their target *S. mutans* isolates by a modified critical dilution method and spot-on-lawn assay as devised by De Vuyst et al. [9] with slight modifications. For this purpose, 100 μl from the overnight culture of the target *S. mutans* isolates were added to the molten soft agar and poured as an overlay to form bilayered trypticase soy agar plate. To the wells punched on the inoculum seeded plates, 50 μl of the diluted and undiluted enterocin were added. The plates were incubated at 37°C for an overnight period in CO₂.
environment and the inhibitory activity of the enterocin was detected by observing the inhibition zones around the wells containing the enterocin.

**Results**

**Isolation of E. faecalis and S. mutans:** Ten isolates of *E. faecalis* were isolated from the stool samples of human subjects. Fifty *S. mutans* isolates were isolated from the people demonstrating high level of caries incidence.

**Qualitative detection of enterocin production:** The inhibitory activity of enterocins produced by *E. faecalis* isolates of *S. mutans* ranged from 30% to 84% (Table 1). *E. faecalis* SF118 and *E. faecalis* OF87 demonstrated highest and lowest inhibitory activity, respectively on *S. mutans* isolates. *E. faecalis* SF118, *E. faecalis* SF101, and *E. faecalis* PF 98 demonstrated inhibitory activity on more than 75% of the indicator *S. mutans* isolates and hence were selected for further analysis.

**Table 1: Inhibitory activity of enterocins on S. mutans**

| S. No | Isolates of E. faecalis | No. of S. mutans inhibited by Enterocin (n=50) | Inhibition percentage of enterocin |
|-------|-------------------------|---------------------------------------------|-----------------------------------|
| 1     | *E. faecalis* SF101     | 39                                         | 78                                |
| 2     | *E. faecalis* SF118     | 42                                         | 84                                |
| 3     | *E. faecalis* GF142     | 29                                         | 58                                |
| 4     | *E. faecalis* PF122     | 36                                         | 72                                |
| 5     | *E. faecalis* OF87      | 15                                         | 30                                |
| 6     | *E. faecalis* SF123     | 24                                         | 48                                |
| 7     | *E. faecalis* GF132     | 27                                         | 54                                |
| 8     | *E. faecalis* PF98      | 38                                         | 76                                |
| 9     | *E. faecalis* PF106     | 30                                         | 60                                |
| 10    | *E. faecalis* SF136     | 18                                         | 36                                |

**Quantitative detection of enterocin activity:** The crude enterocin from three *E. faecalis* isolates with potent anti-*S. mutans* activity was obtained and used in undiluted and diluted concentrations for the quantitative determination of their inhibitory activity against their target *S. mutans* isolates. Among the three enterocins tested, enterocin SF118 exhibited inhibitory activity on 83.3% of the target *S. mutans* isolates at 1:64 dilution whereas, enterocin
PF98 inhibited 73.6% and enterocin SF101 inhibited 66.6% of their target *S. mutans* at the same dilution. Enterocin SF118 and enterocin SF101 inhibited 100% of their target *S. mutans* isolates up to 1:4 dilutions and 1:2 dilutions respectively (Fig. 1).

**Fig. 1:** Inhibitory activity enterocins on *S. mutans* isolates

**Determination of bactericidal activity of enterocin by time kill assay** - The mode of action of the three crude enterocins was determined at its minimum concentration, which inhibited its entire target *S. mutans* isolates by time kill assay. The OD$_{600}$ corresponding to 0.6 was the cell concentration of *S. mutans* suspension before the addition of enterocin. The viable counts of target *S. mutans* isolates determined at 0, 4, 8 and 12 h time interval showed a gradual decline following treatment with enterocins. Among the 3 enterocins tested, enterocin SF118 demonstrated the highest decline in the viability of *S. mutans* isolates with the reduction in the mean log$_{10}$ CFU/ml from 7.79 at 0 h to 4.92 at 12 h of incubation. The least bactericidal activity was observed for enterocin PF98, followed by enterocin SF101 which demonstrated a decline in mean log$_{10}$ CFU/ml from 7.79 at 0 h to 5.97 and 5.33 respectively at the 12 h of incubation. Statistical analysis showed a P-value of 0.001 for the mean log$_{10}$ CFU/ml of *S. mutans* treated with enterocins compared to the untreated control group indicated a significant reduction in the viable count of *S. mutans* isolates following treatment with enterocins.

**Fig. 2:** Mean log$_{10}$ CFU/ml of *S. mutans* following addition of enterocin at various time intervals

**DISCUSSION**

Enterococci are the dominant microflora in the consortium of the human large and small intestine \[14\]. Among the species of Enterococcus, *E. faecalis* predominates in faeces \[15\]. In our study, *E. faecalis* were obtained from faecal specimens and was isolated from all 10 faecal specimens studied.

Dental caries is one of the most common and costly diseases in the world. *S. mutans* plays a central role in the etiology of dental caries by adhering to the enamel surface to produce extracellular polysaccharides, which embroil in cell-cell and cell-surface adhesion instigating bacterial aggregation to create a pre-cariogenic microenvironment. Acid produced by microorganisms in this milieu cause demineralization of tooth structures resulting in dental caries \[16\]. Persons with caries have high levels of *S. mutans* in the oral cavity \[17\]. In the present study, *S. mutans* was isolated from all salivary specimens obtained from people with an incidence of caries.

Lactic acid bacteria comprise at its core *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus*, and *Streptococcus* sp. A great number of organisms of this group produced during their growth, substances of protein structure (either proteins or polypeptides) possessing antimicrobial activities, called bacteriocins. The activity of bacteriocins was restricted to the strains, species or bacteria closely related to the producing species \[18\]. Bacteriocins produced by LAB of *Enterococcus* genus was designated as enterocins and
was frequently characterized among *E. faecalis* and *E. faecium* [6,19]. Fermented foods, environment, clinical pathogens and gastrointestinal tract of humans serve as good niches for isolating bacteriocin producing isolates [20]. In our study enterocin production was demonstrated among the *E. faecalis* isolates obtained from faeces. This study documents the activity of the 10 enterocin producing *E. faecalis* isolates to inhibit *S. mutans*. 3 *E. faecalis* isolates produced potent enterocins that was active to inhibit more than 75% of *S. mutans* used as indicators in our study. In another study, enterocin from *E. faecalis* ESF100 has been reported for its broad spectrum antagonistic property, including inhibition of all 30 *S. mutans* strains tested [21]. The activity of enterocin had been earlier reported on *Listeria*, *Clostridium*, *E. coli* and some viruses [22]. In this study, enterocin SF101 and enterocin SF118 demonstrated inhibitory action on 100% of the target *S. mutans* isolates in its 1:2 and 1:4 dilution respectively whereas, enterocin PF98 showed the similar inhibition percentage at its undiluted form. Previous studies have cited enterocin with minimal inhibitory concentration in the dilutions of 1:128, 1:4 and 1:2, when evaluated against *S. aureus*, *N. meningitidis* and *X. maltophilia* as indicator isolates [21].

In our study, we observed a decline in the viable count of *S. mutans* following the addition enterocins on its target *S. mutans* isolates. The mean log_{10} CFU/ml of *S. mutans* isolates declined from 7.79 at the 0 h of the study of a range of 4.92–5.97 of the 12th hour of enterocin addition. However, the control showed an increase in viable count reaching mean log_{10} CFU/ml of 9.11 at 12 h. The decline in the viable cell count of *S. mutans* validate the ability of enterocins to exert a bactericidal mode of action. In a previously reported study, Enterocin ON-157 produced by *E. faecium* NIAI 157 was found to exhibit bactericidal activity on the target cells. Enterocin ON-157 when added to the growing cells of indicator cultures resulted in a rapid and proportional decrease in the viable count during 240 min incubation period [23].

A study, in which employed a combination of 2 enterocins with 2 antibiotics has earlier reported the reduction in CFU/ml counts of MRSA by 2–3 logs during 3–24 h incubation [24].

**CONCLUSIONS**

With rampant occurrence of caries among all age groups due to modifications in life style practices, impairment to the physical and functional aspects of tooth is on the rise. This necessitates identifying newer solutions to the fading abilities of compounds currently used in combating this problem. The present study documented the possibility of enterocins in reducing the levels of *S. mutans* by bactericidal mode of action. This research had opened avenues for more insights pertaining to the application of enterocins as a potential bio alternative for promoting oral health and reducing the disease burden of caries.

The above study had authenticated the anti-*S. mutans* activity of enterocins. However, studies on a larger sample size employing refined enterocin would expand the horizons of this study to forge ahead this *in-vitro* work into a clinical trial.

**ACKNOWLEDGMENTS**

The authors wish to acknowledge the technical assistance rendered by Mr. Venkatesan and laboratory help of Mr. Raman during this study.

**CONTRIBUTION OF AUTHORS**

Research concept- R. Arularasi Aberna, K. Prabhakar
Research design- R. Arularasi Aberna
Supervision- K. Prabhakar
Data analysis and interpretation- R. Arularasi Aberna
Literature search- R. Arularasi Aberna
Writing article- R. Arularasi Aberna
Critical review- K. Prabhakar
Article editing- R. Arularasi Aberna
Final approval- K. Prabhakar

**REFERENCES**

[1] Subramenium GA, Vijayakumar K, Pandian SK. Limonene inhibits streptococcal biofilm formation by targeting surface-associated virulence factors. J. Med. Microbiol., 2015; 64(8): 879-90. doi: 10.1099/jmm.0.000105.

[2] Marsh PD. Dental plaque as a biofilm and a microbial community-implication for health and disease. BMC Oral health, 2006; 6 Suppl.: S1-S14. doi: 10.1186/1472-6831-6-S1-S14.

[3] Maran S, Shashikiran ND, Aahirwar P, Maran P, Kannojiya PR, Niranjan B. Prevalence of dental caries and traumatic dental injuries among 6-to 12-year-old children in Bhopal City, India. Int. J. Clin. Pediatr. Dent., 2017; 10(2): 172-76. doi: 10.5005/jp-journals-10005-1429.

Copyright © 2015–2018 | ILSSR by Society for Scientific Research under a CC BY-NC 4.0 International License | Volume 04 | Issue 06 | Page 2070
[4] Sicca C, Bobbio E, Quartuccio N, Nicolò G, Cistaro A. Prevention of dental caries: A review of effective treatments. J. Clin. Exp. Dent., 2016; 8(5): e604–e10. doi: 10.4317/jced.52890.

[5] Sheiham A, James WP. Diet and dental caries: the pivotal role of free sugars reemphasized. J. dent. res., 2015; 94(10): 1341-47. doi: 10.1177/0022034515590377.

[6] Foulquie Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L. The role and application of Enterococci in food and health. Int. J. Food Microbiol., 2006; 106(1): 01-24. doi: 10.1016/j.ifoodmicro. 2005.06.026.

[7] Aberna A, Prabakaran K. Evaluation for the association of virulence determinants among E. faecalis with its clinical outcome. Int. J. Biol. Med. Res., 2011; 2(2): 523-27.

[8] Corsetti A, Settanni L, Van Sinderen D. Characterization of bacteriocin-like inhibitory substances (BLIS) from sourdough lactic acid bacteria and evaluation of their in vitro and in situ activity. J. Appl. Microbiol., 2004; 96(3): 521-34. doi: 10.1111/j.1365-2672.2004.02171.x.

[9] De Vuyst L, Moreno MF, Revets H. Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. Int. J. Food Microbiol., 2003; 84(3): 299-318. doi: 10.1016/S0168-1605(02)00425-7.

[10] Hu CB, Malaphan W, Zendo T, Nakayama J, Sonomoto K, et al. A novel two-peptide bacteriocin from Enterococcus faecium KU-BS, has an antibacterial spectrum entirely different from those of its component peptides. Appl. Environ. Microbiol., 2010; 76(13): 4542-45.

[11] Facklam RR, Collins MD. Identification of Enterococcus species isolated from human infections by a conventional test scheme. J. Clin. Microbiol., 1989; 27(4): 731-34.

[12] Mackie TJ. Mackie & McCartney practical medical microbiology. New York, NY, USA: Churchill living stone; 1989.

[13] Del Campo R, Tenorio C, Jimenez-Diaz R, Rubio C, Gomez-Lus R, et al. Bacteriocin Production in Vancomycin-Resistant and Vancomycin-Susceptible Enterococcus Isolates of Different Origins. Antimicrob. Agents Chemother., 2001; 45(3): 905-12. doi: 10.1128/AAC.45.3.905-912.2001.

[14] Hayashi H, Takahashi R, Nishi T, Sakamoto M, Benno Y. Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. J. Med. Microbiol., 2005; 54(11): 1093-1101.

[15] Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, et al. Diversity of the human intestinal microbial flora. Sci., 2005; 308(5728): 1635–38. doi: 10.1126/science.1110591.

[16] Forsten SD, Bjorklund M, Ouwehand AC. Streptococcus mutans, caries and simulation models. Nutrients, 2010; 2(3): 290-98.

[17] Soderling EM. Xylitol, mutans streptococci, and dental plaque. Adv. Dent. Res., 2009; 21(1): 74-78.

[18] Zacharof MP, Lovitt RW. Bacteriocins produced by lactic acid bacteria a review article. APCBEE Procedia, 2012; 2: 50-56. doi: 10.1016/j.apcbee.2012.06.010.

[19] Javed I, Ahmed S, Ali MI, Ahmad B, Ghumro PB, Hameed A, Chaudry G. Bacteriocinogenic potential of newly isolated strains of Enterococcus faecium and Enterococcus faecalis from dairy products of Pakistan J. Microbiol. Biotechnol., 2010; 20(1): 153-60.

[20] Nes IF, Diep DB, Ike Y. Enterococcal, Bacteriocins and Antimicrobial Proteins that Contribute to Niche Control. In Gilmore MS, Clewell (ed), Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. Massachusetts Eye and Ear Infirmary, Boston, 2014.

[21] Ahmad SA, Iqbal A, Rasool SA. Isolation and biochemical characterization of enterocin ESF 100 produced by Enterococcus faecalis ESF 100 isolated from a patient suffering from urinary tract infection. Pak. J. Bot., 2004; 36(1): 145-58.

[22] Foulquie-Moreno MR, Callewaert R, Devreese B, Van Beeumen J, et al. Isolation and biochemical characterisation of enterocins produced by Enterococci from different sources. J. Appl. Microbiol., 2003; 94(2): 214-29.

[23] Ohmomo S, Murata S, Katayama N, Nitisinsprasart S, Kobayashi M, et al. Purification and some characteristics of enterocin ON-157, a bacteriocin produced by Enterococcus faecium NIAI 157. J. Appl. Microbiol., 2000; 88(1): 81-89. doi: 10.1046/j.1365-2672.2000.00866.x.
[24] Al Atya AK, Belguesmia Y, Chataigne G, Ravallec R, Vachee A, et al. Anti-MRSA activities of enterocins DD28 and DD93 and evidences on their role in the inhibition of biofilm formation. Frontiers in Microbiol., 2016; 31(7): 817.