

# Enterocins: Symptomatic for Bio-alternative in Caries Control

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## 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* isolates 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 log<sub>10</sub> 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:** Enterocin, *S. mutans*, Inhibitory activity, Dental caries

## 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

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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 enterocinogenic 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 Rajah Muthiah Institute of Health sciences, Annamalai University were 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 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 salivary 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 produce enterocins which would inhibit *S. mutans* (indicator isolates) were detected by agar spot assay as 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 CO<sub>2</sub> environment. An isolate was considered as an enterocin producer when an inhibition zone of the indicator isolate was found around any of the test isolate.

**Quantitative detection of enterocin activity-** Three isolates of *E. faecalis* which produced enterocin showing 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 CO<sub>2</sub> environment and the inhibitory activity of the enterocin was detected by observing the inhibition zones around the wells containing the enterocin.

**Determination of bactericidal activity of enterocin by time kill assay-** Time kill assay was performed to detect the suitability of the enterocin for biomedical application in reducing the *S. mutans* levels. The enterocins were tested at their minimum concentration that inhibited all its target isolates. *S. mutans* suspension in BHI broth at its log phase of growth with its OD<sub>600</sub> corresponding to 0.6 was used as inoculum. For each enterocin tested, 5ml of the inoculum was dispensed aseptically to eight sterile test tubes. 0.5ml of enterocin was added to the first set of four test tubes. The rest four test tubes for each isolate containing the *S. mutans* alone served as controls. All the test and control tubes were incubated at 37°C in CO<sub>2</sub> environment and at varying time intervals of 0, 4, 8 and 12 h; a test and control tube for each isolate was taken to determine the viable count of *S. mutans*.

**Viable count determination-** The viable count of *S. mutans* following enterocin treatment was determined by spread plate technique. At specified time intervals of the study, 100 µl of each *S. mutans* isolated from the test and control tubes were diluted in sterile saline and 100 µl of the diluted sample was placed onto the surface of sterile, dry nutrient agar plates. The inoculum was spread using L-rod to ensure a lawn culture and allowed to dry. The plates were incubated at 37°C in CO<sub>2</sub> environment for 24 h and the CFU/ml was determined. The decline in the CFU/ml of *S. mutans* after the addition of enterocin against time indicates its bactericidal potential and hence its possibility for biomedical application.

**Statistical Analysis-** The mean log<sub>10</sub> CFU/ml were analyzed and compared with one-way ANOVA test and Students 't' test using SPSS software 16 version. P-value<0.05 at 5% level of significance was considered as statistically significant.

## 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 people demonstrating high level of caries incidence.

**Qualitative detection of enterocin production-** The inhibitory activity of enterocins produced by *E. faecalis* isolates on *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.

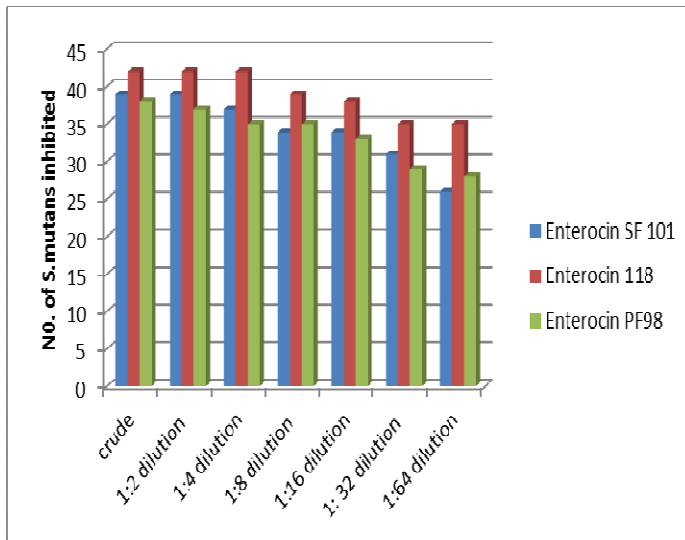
**Table 1: Inhibitory activity of enterocins on *S. mutans***

S. No.	Isolates of <i>E. faecalis</i>	No. of <i>S. mutans</i> inhibited by Enterocin (n=50)	Inhibition percentage of enterocin
1	<i>E. faecalis</i> SF101	39	78
2	<i>E. faecalis</i> SF118	42	84
3	<i>E. faecalis</i> GF142	29	58
4	<i>E. faecalis</i> PF122	36	72
5	<i>E. faecalis</i> OF87	15	30
6	<i>E. faecalis</i> SF 123	24	48
7	<i>E. faecalis</i> GF132	27	54
8	<i>E. faecalis</i> PF 98	38	76
9	<i>E. faecalis</i> PF106	30	60
10	<i>E. faecalis</i> 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 quantitative determination of their inhibitory activity against their target *S. mutans* isolates.

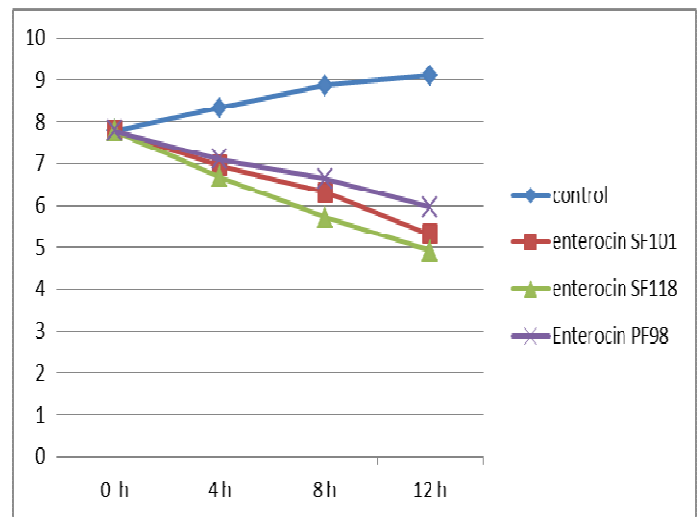
Among the three enterocins tested, enterocin SF118 exhibited inhibitory activity on 83.3% of the target *S. mutans* isolates at 1:64 dilution whereas, enterocin PF98 inhibited 73.6% and enterocin SF101 inhibited 66.6% of their target *S. mutans* at the same dilution.

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<sub>600</sub> 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<sub>10</sub> 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<sub>10</sub> 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<sub>10</sub> CFU/ml of *S. mutans* treated with enterocins compared to the untreated control group indicating a significant reduction in the viable count of *S. mutans* isolates following treatment with enterocins.



**Fig. 2: Mean log<sub>10</sub> CFU/ml of *S. mutans* following addition of enterocin at various time intervals**

## DISCUSSION

Enterococci are the dominant microflora in the consortium of 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 ten 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*. A great number of organisms of this group produce during their growth, substances of protein structure (either proteins or polypeptides) possessing antimicrobial activities, called bacteriocins. The activity of bacteriocins is restricted to the strains, species or bacteria closely related to the producing species [18]. Bacteriocins produced by LAB of *Enterococcus* genus are designated as enterocins and are 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 ten enterocin producing *E. faecalis* isolates to inhibit *S. mutans*. Three *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 the 30 *S. mutans* strains tested [21]. The activity of enterocin has 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 at 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<sub>10</sub> CFU/ml of *S. mutans* isolates declined from 7.79 at the 0 h of the study to a range of 4.92 - 5.97 by the 12<sup>th</sup> hour of enterocin addition. However, the control showed an increase in viable count reaching mean log<sub>10</sub> CFU/ml of 9.11 at 12 h. The decline in the viable cell count of *S. mutans* validates 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 which employed a combination of 2 enterocins with 2 antibiotics had 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 rise.

This necessitates identifying newer solutions to the fading abilities of compounds currently used in combating this problem. The present study documents the possibility of enterocins in reducing the levels of *S. mutans* by bactericidal mode of action. This research has 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 has 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.

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## 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

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