Antibacterial Effects of Effective Ecoproduce on *Enterococcus faecalis*: An in vitro Study

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Abstract
Sodium hypochlorite, the gold-standard endodontic irrigant, has several disadvantages. Effective ecoproduce (EEP) is an organic product made from kitchen waste, molasses, and water and a natural cleansing agent. We sought to determine the antibacterial activities of EEP with varying source materials and fermentation periods. EEP was prepared from pineapple (P), orange (O), and a mixture of pineapple and orange (M) and fermented for 3 and 6 months. The minimum inhibitory concentration (MIC) of EEP toward *Enterococcus faecalis* was determined via broth microdilution assay and turbidity measurements. The minimum bactericidal concentration (MBC) was also determined. The MIC value was 50% for MEEP at 3 months and for OEEP and MEEP at 6 months of fermentation. Meanwhile, the MIC value was 100% for 3 months old PEEP - and OEEP. An MBC of 100% was detected for OEEP and MEEP at 3 months of fermentation. The variation in source materials (p = 0.021) but not fermentation periods (p = 0.243) provoked a statistically significant effect on the antibacterial properties of EEP. EEP of different source materials with various fermentation periods has antibacterial effects toward *E. faecalis*.

Keywords: Waste management, garbage, minimum inhibitory concentration, *Enterococcus faecalis*, antibacterial.


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Introduction
Effective ecoproduce (EEP) is a product of organic solid kitchen wastes, involving 10 parts water, 3 parts kitchen wastes (vegetables and/or fruit peels), and 1 part brown sugar/molasses, fermented for, ideally, 3 months in an air-tight plastic container.¹ First created by Dr Rosukon Poompanvong, a Thai farmer, it is more commonly known as “garbage enzyme.” EEP is typically used as fertilizer, insecticide, detergent and for skin care, sludge management, and water treatment¹,², owing to some of its properties mentioned below.

Arun and Sivashanmugam (2015) previously reported the protease, lipase, and amylase activities of EEP made of tomato, cauliflower, pineapple, orange, and mango dregs, fermented for 3 months.³ In a separate study, the same authors revealed that different types of kitchen waste produce EEP samples that vary in terms of hydrolytic enzymatic activities. They concluded that an EEP of pineapple peels:orange peels in a 6:4 ratio possesses higher degree of hydrolytic enzyme activity. In relation to the sludge solubilization potential, pineapple EEP and orange EEP showed better performance relative to that of tomato EEP, cauliflower EEP, and mango EEP, with slightly higher reduction rates in volatile suspended solids (VSS) and total suspended solids (TSS) by 20% to 25% and higher rates of solubilization of soluble chemical oxygen demand (COD), soluble total phosphorus (TP) and soluble total Kjeldhal nitrogen (TKN) by 20% to 25%, 9% to 11%, and 15% to 20%, respectively.

Besides, EEP is known to contain various organic acids, in which acetic acid was shown to have the highest concentration at 3 months of fermentation when compared with other acids such as citric acid, malic acid, oxalic acid, and lactic acid, all detected through reserved phase-

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high performance liquid chromatography (RP-HPLC). Further, some studies reported the presence of antibacterial properties of EEP toward various pathogens like Escherichia coli, Staphylococcus aureus, Salmonella typhi, Candida albicans, Streptococcus pyogenes, and Aspergillus niger. The disinfectant potential of EEP was also proven using phenol coefficient, whereby EEP can be diluted 4 times as much as phenol and yet has an equivalent killing power for S. typhi to that of phenol.3

On the other hand, the characteristics of EEP vary with time. Nazim and Vasudevan (2017) reported that EEP solution was not ideal for treating wastewater immediately after filtering from its solids—rather, it is most effective at 60 days after filtration.2

Enterococcus faecalis (E. faecalis) is commonly found in persistent root canal infections. It is a Gram-positive coccus that normally inhabits the oral cavity but which can occur in posttreatment apical periodontitis. E. faecalis is able to survive the harsh conditions in the root canal system, owing to its multiple virulence factors.10

On top of canal shaping, effective irrigation of the root canal is important to eliminate intracanal pathogens. While the gold-standard irrigant has always been sodium hypochlorite (NaOCl), such has been reported to have potential adverse effects on the patient and operator alike.6 If extruded into the soft tissue beyond the apical foramen, NaOCl may cause swelling and facial hematoma as it is toxic to human tissues at bactericidal concentrations.6 Further, it also has a foul smell and decolorizes clothing easily when spillage occurs.6

Due to the existing problems of NaOCl, there have been many attempts by different researchers to develop an alternative irrigant able to overcome the shortcomings of NaOCl but which, at the same time, can eliminate the microflora in the root canal, most of which are plant-based.11–14 One possible option is to use effective ecoproduce (EEP).

Given this, the current study sought to investigate the antibacterial properties of EEP having different source materials and fermentation periods against E. faecalis. It was hypothesized that there would be no difference found in the degree of antibacterial efficacy of EEP with such variations on E. faecalis.

Materials and methods

1.1 Preparation of EEP

To compile the EEP, we employed a modification of Prakash’s method, whereby 75 g of pineapple peels, 25 g of molasses, and 250 mL of water were mixed together in an air-tight plastic container with a capacity of 700 mL. The mixture was then covered tightly with the lid and left for 3 months to allow for fermentation, which was continued on until 6 months of fermentation where stipulated. The containers were opened once a day for 10 seconds to release trapped gas.1

Other EEP concoctions were prepared by replacing the pineapple peels in the above steps with either orange peels or a mixture of 45 g of pineapple peels and 30 g of orange peels. Each sample with different source materials was prepared at 3 different times to create 3 biological triplicates. However, the same type of fruit was brought from the same vendor each time to minimize variability in this study.

At 3 months and 6 months of fermentation, the EEP from all 3 sources—namely, pineapple (P), orange (O), and a mixture of pineapple and orange in a 6:4 ratio (M)—were sterilized using a 0.2-µm pore-sized syringe filter (Minisart®; Germany) and employed as test samples.

1.2 Bacterial strain

Frozen E. faecalis ATCC® 29212™ (American Type Culture Collection, Manassas, VA, USA) stock was revived by thawing at room temperature for 5 minutes. Then, 100 µL of the thawed stock was pipetted and streaked onto brain heart infusion (BHI) agar. Another 100 µL of the thawed E. faecalis was transferred into 10 mL of BHI broth premeasured in a universal bottle. Both cultures were incubated (Lab Companion, Korea) anaerobically for 24 hours at 37°C and with 95% relative humidity.

After 24 hours of incubation, bacteria purity was confirmed by Gram staining and the observation of growth colony on agar and of growth patterns in the broth.17 The overnight bacterial culture was diluted 100 times with BHI broth to achieve a standardized suspension of OD0.25nm = 0.03 on the spectrophotometer (JENWAY®; Cole-Parmer, Vernon Hills, IL, USA).18 The prepared bacterial suspension was used for assays within 15 minutes of preparation.

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as suggested by the Clinical and Laboratory Standards Institute (CLSI).\(^\text{19}\)

1.3 Minimum inhibitory concentration (MIC) assay

The broth microdilution assay was carried out according to the CLSI protocol\(^\text{19}\) with slight modifications, at 3 months and 6 months of EEP fermentation, respectively. Briefly, all 3 different source materials (P, O, and M) of EEP—which were already sterile-filtered—were serially diluted using BHI broth to produce concentrations of 0.78%, 1.56%, 3.13%, 6.25%, 12.5%, 25%, 50%, and 100%.\(^\text{20}\)

The diluted EEP samples were then seeded into a 96-well plate (HmbG, Malaysia) by pipetting 50 µL of pineapple-based EEP into each well, in triplicate. The seeding of EEP was then repeated with the orange-based EEP and mixed-material EEP.

Next, 50 µL of the prepared bacterial suspension of \textit{E. faecalis} was pipetted into the wells preloaded with EEP, which brought the final concentration of bacteria in each well to \(10^5\) colony-forming units (CFU)/mL.\(^\text{19}\)

The assay was also applied to control wells with co-incubation of the following:

(i) Positive control: 50 µL of bacterial suspension and 50 µL of 5.25% NaOCl (Clorox®; The Clorox Company, Oakland, CA, USA)

(ii) Negative control: 50 µL of bacterial suspension and 50 µL of BHI broth

(iii) Untreated controls: 50 µL of EEP (each concentration per well) and 50 µL of BHI broth

All test and control samples were done in triplicate and incubated anaerobically for 24 hours at 37°C. After 24 hours of incubation, the turbidity of each well was observed with the naked eye, comparing the appearance of black lines established through the test wells and control wells.\(^\text{21}\) Turbid wells meant that there was positive growth of bacteria, whereas clear wells meant that there was no growth of bacteria. The MIC was confirmed quantitatively as the turbidity of each well was measured through optical-density readings using an enzyme-linked immunosorbent assay plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 590 nm.\(^\text{20}\) The MIC was determined by comparing the turbidity and absorbance of the suspension in test wells with that of the corresponding untreated control wells.\(^\text{22}\) The lowest concentration of EEP at which there was no visible growth noted in the test wells was characterized as the MIC.\(^\text{19}\)

1.4 Minimum bactericidal concentration (MBC) test

Samples from wells with negative microbial growth (i.e., clear wells) as seen during the MIC assay were subcultured on BHI agar plates at 37°C for 24 hours under anaerobic conditions. MBC was defined as the lowest concentration of EEP that showed no bacterial growth.\(^\text{23}\) MBC testing was conducted for EEP of all source material compositions at 3 months and 6 months of fermentation.

1.5 Data recording and analysis

The MIC and MBC values of each source material at 3 and 6 months of fermentation periods were observed and recorded. At the same time, the difference between the optical density (OD) of the test and control wells was calculated using the following formula:

\[
\text{Mean difference in OD (ΔOD)} = \frac{(\text{OD}_{\text{test wells in 1st tritlicate}} - \text{OD}_{\text{control wells in 1st tritlicate}}) + (\text{OD}_{\text{test wells in 2nd tritlicate}} - \text{OD}_{\text{control wells in 2nd tritlicate}}) + (\text{OD}_{\text{test wells in 3rd tritlicate}} - \text{OD}_{\text{control wells in 3rd tritlicate}})}{3}
\]

The MICs for P-EEP, O-EEP, M-EEP at 3 and 6 months of fermentation were then confirmed following statistical comparison of the ΔOD for each concentration with that of the positive (NaOCl) and negative (BHI broth) controls. One-way analysis of variance (ANOVA) in the Statistical Package for the Social Sciences (SPSS) version 23 software program (IBM Corp., Armonk, NY, USA) was used for normally distributed and homogenous data, whereas Welch’s ANOVA was conducted for normally distributed data that violated the homogeneity of variance.

Finally, a 2-way mixed ANOVA was run to see whether different source materials and fermentation periods have an effect on the antibacterial activity (MIC) of EEP.
Results

2.1 pH of EEP
The original pH (at 100% concentration) of all EEP samples was 4.3 ± 0.11.

2.2 MIC and MBC through inspection with the naked eye

The findings of MIC and MBC tests are listed in Table 1.

![Figure 1. ∆OD for all controls and concentrations in 3-months-old EEP of various source materials.](image1)

![Figure 2. ∆OD for all controls and concentrations in 6-months-old EEP of various source materials.](image2)

<table>
<thead>
<tr>
<th>Fermentation period (months)</th>
<th>Three</th>
<th>Six</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pineapple</td>
<td>Orange</td>
<td>Mixed</td>
</tr>
<tr>
<td>MIC</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>MBC</td>
<td>-</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 1. MIC and MBC values of EEP observed with the naked eye.

Note: MIC and MBC values are based on the percentage of concentration of EEP.

2.3 MIC determination via statistical analysis

Apart from 3-months-old O-EEP (one-way ANOVA, followed by post-hoc Tukey HSD analysis), all the other EEP samples made from different source materials aged 3 and 6 months old underwent Welch’s ANOVA for MIC determination, as their data had no significant outliers (as assessed by boxplot) and were normally distributed (Shapiro–Wilk test: p > 0.05) but violation of homogeneity was observed (Levene's test of homogeneity of variance: p < 0.05). This was followed by post-hoc Games–Howell analysis.

Figures 1 and 2 show the ∆OD for all controls and concentrations in each tested source material at 3 and 6 months of fermentation, respectively. For a certain concentration to be defined as the MIC, the ∆OD was required to show no statistically significant difference relative to that of the positive control’s ∆OD and, at the same time, have a statistically significant difference with that of the negative control’s ∆OD. MICs determined after statistical analyses are marked with “a” in Figure 1 and Figure 2. It was concluded that the statistical analysis of ∆OD confirmed the naked-eye observation of MIC, as the MIC of each group corresponded with that observed with the naked eye.

2.4 Effects of source material and fermentation period on the antibacterial potential of EEP

A 2-way mixed ANOVA was run to see whether different source materials and fermentation periods played a role in the antibacterial effects of EEP, which was expressed through ∆OD (or MIC). As 6-months-old pineapple EEP did not have an MIC, only the ∆OD of orange and mixed-material EEP of both fermentation periods were taken into account during this statistical analysis.

There were no outliers as assessed by boxplot. The data were normally distributed as assessed by the Shapiro–Wilk test of normality (p > 0.05). There was homogeneity of variances (p > 0.05) and covariances (p > 0.05) as assessed by Levene’s test of homogeneity of variances and Box’s M test, respectively. The results of the 2-way mixed ANOVA and the subsequent main effects of fermentation period and source material as well as post-hoc analyses are reported in Table 2. It was hence concluded that
different source materials of EEP had statistically significant different antibacterial effects, while EEP of different fermentation periods showed no statistically significant difference in terms of such.

<table>
<thead>
<tr>
<th>Two-way mixed ANOVA</th>
<th>Fermentation period</th>
<th>Source material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction between fermentation period and source material</td>
<td>F = 0.18, p = 0.69</td>
<td></td>
</tr>
<tr>
<td>Simple main effect</td>
<td>F = 1.87, p = 0.24</td>
<td>F = 13.66, p = 0.02*</td>
</tr>
</tbody>
</table>

Table 2. Results of 2-way mixed ANOVA, the subsequent main effect of fermentation period and source material, and post-hoc analysis.

* p is statistically significant at α = 0.05
† mean difference (mean ± standard error)
ANOVA: analysis of variance; SD: standard deviation

**Discussion**

From the results, we deduced that EEP fermented for 3 months was better than that fermented for 6 months as the latter did not exhibit a bactericidal effect toward *E. faecalis*. From our observation, O-EEP and M-EEP at 3 months of fermentation in particular had the potential to kill planktonic *E. faecalis*. Bacteriostatic effects were seen at a lower concentration (50%) for 3-months-old M-EEP as compared with O-EEP, which only exhibited MIC at the original (100%) concentration.

Statistical analyses revealed that antibacterial potential varies between different EEP source materials, while different fermentation periods did not show such an outcome. Therefore, the hypothesis that stated that there is no difference in the MIC and MBC of EEP on *E. faecalis* with source materials and fermentation periods is rejected.

In a prior antibacterial study using a disc-diffusion method, Arun and Sivashanmugam7 brewed EEP from a mixture of tomato, cauliflower, pineapple, orange, and mango peels, then reported that, at the tested concentrations of 5%, 10%, and 15% EEP, the growth rates of *E. coli*, *S. aureus*, *S. typhi*, and *C. albicans* were inhibited. More interestingly, the authors also noted that EEP had a higher inhibitory effect when it had a pH of 7 in comparison with the original acidic pH of 3.6 as the enzymatic activities were optimal at a neutral pH, causing lysis to the tested pathogens.

As observed in our study, the original pH of all EEP samples was 4.3 ± 0.11. The pH values of all tested samples of EEP were not standardized throughout the experiment as the EPP pH values increased as they were diluted with BHI broth to lower concentrations. At a 6.25% concentration and below, the EEP pH was observed to be in the neutral range—that is, between 7 and 7.38.

In a separate study, Saramanda and Kaparapu5 also reported their findings about the antibacterial effects of lemon peel–based EEP against some bacteria (*E. coli*, *S. aureus*, *S. pyogenes*, *S. typhi*, and *Pseudomonas aeruginosa*), and fungus (*A. niger*, *Fusarium spp.*, and *Cladosporium spp.*). They also employed a disc-diffusion method to test the antibacterial activity of the lemon peel EEP against the tested pathogens and compared the zone of inhibition with that of various positive controls. These authors reported that the zones of inhibition (measured in millimeters) of all the tested microorganisms were higher than that of the positive controls, which means that the lemon peel EEP had better antibacterial properties against the tested pathogens.

The methodology (broth microdilution assay) for MIC and MBC assays in the present study was conducted in accordance with the relevant CLSI guidelines19,24 with slight modifications and reference to the European Committee for Antimicrobial Susceptibility Testing (EUCAST) guidelines.21

According to Balouiri et al. (2016),20 the broth microdilution method is reproducible and economical as it involves a small volume of broth dispensed in sterile 96-well plates and does not consume significant space in the laboratory. The broth macrodilution method, on the other hand, requires large amounts of reagent and space.20 As the final bacterial concentration in each well plays a role in the determination of the MIC values20,25, the diluted bacterial suspensions were seeded into the test wells within 15 minutes of preparation to achieve the standardized final concentration of 105 CFU/mL of bacteria in each test well, per the CLSI standard.19 To ensure the MIC determination was not hampered by toxic medium, BHI, which was used in this study, was pretested to be nontoxic toward *E. faecalis* and, as a matter of fact, its use was also recommended by EUCAST.21
EEP was not prepared in sterile conditions because, without the presence of microorganisms, the fermentation of EEP could not take place. In order to avoid a false-negative result (turbid wells at endpoint), all EEP samples were sterile-filtered prior to the assays.\(^{19}\)

It is believed that acetic acid (commonly known as vinegar) played a role in the antibacterial activity of EEP toward \textit{E. faecalis}. Although no tests were conducted to determine the amount of acetic acid in EEP in the current study, it is strongly believed that this compound was present in the crude EEP solution of various source materials and fermentation periods. This is believed because Soo (2010) reported that the actual amount of acetic acid in 50 mL of EEP was calculated to be 3650.36 ppm, detected through the acid–base separation of acetic acid from 3-months old EEP composed of fruit and vegetable waste.\(^{26}\) In addition, Arun and Sivashanmugam\(^{4}\) have shown in their study that a maximum concentration of 78.14 g/L of acetic acid was detected through reverse-phase high-performance liquid chromatography (RP-HPLC) in 3-months-old EEP composed of a mixture of pineapple, orange, tomato, cauliflower, and mango dregs. In addition, lactic acid, malic acid, oxalic acid, and citric acid were among the other organic acids detected. However, unlike acetic acid, these weak acids in EEP experienced a drop in their concentrations from Day 15 of fermentation onwards.\(^{4}\)

Acetic acid is thought to exert its toxic effects toward bacteria through a series of mechanisms as explained by Halstead et al. (2015), Hughes and Webber (2017), and Kundukad et al. (2017).\(^{27-29}\) According to Bjarnsholt et al. (2014), pH 4.76 is the \(pK_a\) of acetic acid.\(^{30}\) Acetic acid diffuses across bacterial cell membranes readily and acidifies the cytoplasm of bacteria that originally presents as neutral (around pH of 7.6) as the pH intracellularly is beyond the \(pK_a\) of acetic acid, causing it to disassociate in the cytoplasm. The accumulation of acids (\(H^+\)) in the bacterial cytoplasm creates an osmotic effect on the bacterial cells, causing an influx of water into the bacterial cells, which increases the turgor pressure and causes lysis; meanwhile, the acetate resulting from the disassociation leads to an array of harmful events such as the disruption of metabolic activities, denaturing of proteins, and DNA damage of the bacteria.

Bjarnsholt et al. (2014) reported the antibiofilm activities of acetic acid against some bacteria (i.e., \textit{S. aureus} and \textit{P. aeruginosa}). They also tested the antimicrobial efficacy of acetic acid in combination with the usage of some antibiotics (e.g., tobramycin, ciprofloxacin, and colistin), observing synergistic effects of such.\(^{30}\)

Elsewhere, Halstead et al. (2015) determined that acetic acid possesses antibacterial effects towards \textit{P. aeruginosa} and \textit{Acinetobacter baumannii} at the MIC value of 0.16\% to 0.31\%.\(^{23}\) Hydrolytic enzymatic activities demonstrated by EEP such as protease, amylase, and lipase activities were described by Arun and Sivashanmugam.\(^{3}\) However, their role in eliminating planktonic bacteria as in this study may be reduced as the pH at the MIC value is rather low (pH 4.37–4.70) and not optimal for enzymatic activities; plus, many papers have discussed the enzymatic role noted in biofilm removal rather than planktonic bacteria elimination.\(^{31,32}\)

To summarize, at a pH of less than 4.76 (\(pK_a\) value of acetic acid), which in our case is also the pH range for the MICs of all EEP samples tested in this study, it was postulated that EEP was able to exert its bacteriostatic/bactericidal effects through the mechanism of action of acetic acid that is present in it. As the concentration of the EEP decreased, the pH of the EEP also increased, eventually exceeding the \(pK_a\) of acetic acid, leading to dissociation of acetic acid and being unable to bring to play its antibacterial effect. On the contrary, the hydrolytic enzymes present in the EEP were not able to function as the pH of the EEP was not optimal for facilitating enzymatic reaction at the MIC level. Besides, in a planktonic state, there are no protein interactions between bacterial cells so, even at an optimal pH in less-concentrated EEP, enzymatic reactions to degrade the bacteria have little role to play. The limitations of the current study are:

(i) The exact active ingredients that are working and playing their roles in causing bacteriostatic and bactericidal effects were not investigated

(ii) The biocompatibility of EEP toward human tissues was not studied

With the results of this research, it is justifiable to suggest future studies like cytotoxicity tests, RP-HPLC, anti-biofilm tests,
and so on must be carried out to investigate the potential of EEP as an endodontic irrigant.

**Conclusions**

EEP concoctions of different source materials and fermentation periods have antibacterial effects toward *E. faecalis*, but 3-months-old EEP created from mixed source materials (pineapple/orange = 6:4) showed the most promising potential as an alternative endodontic irrigant. Future studies are necessary to verify the potential of EEP as an endodontic irrigant, as it is a novel application.

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**Declaration of Interest**

We declare that we have no conflicts of interest to disclose.

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