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RESEARCH ARTICLE

The acid tolerance response and pH adaptation of *Enterococcus faecalis* in extract of lime *Citrus aurantiifolia* from Aceh Indonesia [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: The objective of the present study was to evaluate the acid tolerance response and pH adaptation when *Enterococcus faecalis* interacted with extract of lime (*Citrus aurantiifolia*).

Methods: We used *E. faecalis* ATCC 29212 and lime extract from Aceh, Indonesia. Both materials were analyzed for their pH adaptation, acid tolerance response, adhesion assay, and mass profiles using a light microscope with a magnification of x1000. Further, statistical tests were performed to analyze both correlation and significance of the acid tolerance and pH adaptation also the interaction activity.

Results: *E. faecalis* was able to adapt to a very acidic environment (pH 2.9), which was characterized by an increase in its pH (reaching 4.2) at all concentrations of the lime extract ($p < 0.05$). *E. faecalis* was also able to provide acid tolerance response to lime extract based on spectrophotometric data (595 nm) ($p < 0.05$). Also, the interaction activity of *E. faecalis* and the lime extract was relatively stable within 6 up to 12 hours ($p < 0.05$), but it became unstable within 24–72 hours ($p > 0.05$) based on the mass profiles of its interaction activity.

Conclusions: *E. faecalis* can adapt to acidic environments (pH 2.9–4.2); it is also able to tolerate acid generated by *Citrus aurantiifolia* extract, revealing a stable interaction in the first 6–12 hours.

Keywords

Enterococcus faecalis, Lime (*Citrus aurantiifolia*) extract, acid tolerance, pH adaptation

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Introduction

Enterococcus faecalis is a significant agent in the pathogenesis of root canal infections, especially in post-endodontic treatment, with a prevalence of 24–77% in these infections¹. *E. faecalis* is very difficult to eliminate because the pathogen can survive in poor nutrient conditions. It can adapt to acidic conditions, including living in the dentin tubule of a closed root canal with a smear layer. It can also express the dominant biofilm protein to maintain its attachment to host cells².

E. faecalis has been shown tolerate to acidic environments as well as to adapt to pH changes, which are the essential virulence factors in maintaining antibacterial balance³. Fisher reported that *E. faecalis* could survive in environments with high NaCl concentrations at extreme temperatures of 5–65°C with a pH of 4.5–10.0⁴. Stuart *et al.*¹ reported that *E. faecalis* are less sensitive, with a pH of 5.0 at 25°C after it has been incubated for 10 h. The author also found that it has an excellent growth capability at pH 8.5 and low adhesion at pH 7.1 in a medium coated with bovine serum albumin (BSA).

E. faecalis is resistant to medication materials such as calcium hydroxide⁵ and chlorhexidine (CHX)⁶. The long-term use of both medication materials can lead to parachloroaniline (PAC), causing blockage of the dentinal tubules and eventually becoming toxic⁷. Fosfomycin may also interfere with acid tolerance systems and pH changes of *E. faecalis* in tooth root canals by inhibiting phosphoenolpyruvate synthetase⁸.

Indonesia, especially in Aceh has a tropical climate with a variety of plants that can be utilized in medical treatment, including lime extract (*Citrus aurantiifolia*). It contains phenols, flavonoids, hydrogen peroxide, tannins, alkaloids, and saponins that have antibacterial, antioxidant, antifungal, analgesic, and anti-inflammatory properties⁹. Nwankwo¹⁰ reported that lime extract helped to prevent *Klebsiella pneumonia*, *Salmonella*, and *Escherichia coli*. Here, the acid in lime extract influenced the bacterial development and cell metabolism. The present study evaluates the acid tolerance response and pH adaptation of *E. faecalis* when it interacts with lime extract.

Methods

Materials

The lime extract and *E. faecalis* (ATCC-29212) were used in this study. The extractions were prepared at the Laboratory of Microbiology at the Faculty of Veterinary, University of Syiah Kuala, Darussalam, Banda Aceh, Indonesia. Both materials were made *in vitro* to analyze the pH adaptation, acid tolerance response, and interaction activity between *E. faecalis* and lime extracts.

Lime extraction

Lime peel was separated from the flesh then dried using dehydrator until the water content reduced to 10%. Dried lime peel was grinded into powders. The powder was put into glass container and masserated with ethanol 70% for two days and then

strained using a gauze. Filtrate was evaporated using a rotary evaporator at 80°C to obtain the pure lime extracts.

Culture of *E. faecalis* bacteria

E. faecalis ATCC 29212 taken from glycerol stock was cultured on a Mueller-Hinton Agar (MHA) medium at a temperature of 80°C (Thermo Fisher Scientific Inc., Paisley, UK). The culture was incubated in anaerobic conditions at 37°C for 48 hours using Anaerogen TM GasPack (Oxoid, Basingstoke UK), and incubator (Mettler, Germany). A colony of *E. faecalis* bacteria was subsequently re-cultured in 5 ml of Mueller-Hinton Broth (MHB) medium (Thermo Fisher Scientific Inc, Paisley, UK) in anaerobic conditions at a temperature of 37°C for 48 hours. Afterward, the *E. faecalis* grown on the liquid medium was synchronized further with McFarland 0.5 (1 x 10⁸ CFU/ml) (TM50, Dalynn Biological Inc., Calgary, Canada). The Accuracy of the density of McFarland standart can be checked using a spectrophotometer with an absorbance reading of 0.08 to 0.1 at 625 nm¹¹.

Adaptation to pH assay

A total of 50 ml of lime extracts in several different concentrations (100%, 75%, 50%, 25%, 12.5%, and 6.25%) was placed into different beaker glasses. Then, 5 ml of *E. faecalis* in MHB (1:10) were added to each of the beakers. The initial pH of the mixture was measured (0 hours) before incubation. Next, each beaker was incubated at 37°C for 6 hours, 12, hours, 24 hours, 48 hours, and 72 hours in an anaerobic atmosphere using Anaerogen TM GasPack (Oxoid, Basing stoke UK), at each of these times, the beakers' pH was measured using a pH meter (Thermo Fisher Scientific Inc, Paisley, UK). Various changes in pH from 0 hours to the specified time can be used as an indicator of whether *E. faecalis* has a tolerance response to the acidic environment and can adapt to changing pH¹².

Acid tolerance assay

The cultures of the pH measurements were used to measure the acid tolerance response of *E. faecalis* to lime extract utilizing the principle of spectrophotometry¹³. The analysis was performed based on the incubation time that had been determined following the measurement of pH shaken at 500 rpm. Here, 96 wells of the triple microplate series were coated with 50 µl of MHB (Thermo Fisher Scientific Inc., Paisley, UK) for 15 minutes and then were vacuumed. After that, 100 µl of the test materials (*E. faecalis* + lime extract) derived from the incubation processes at 6 h, 12 h, 24 h, 48 h, and 72 hours were added to each well. Each well was incubated for 15 minutes to analyze the adaptation of the test materials. Then bio-tolerant activity was measured using the Elisa Reader (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 595 nm.

Adhesion assay

Adhesion assay was analyzed based on the principles of Gram-staining¹⁴. The standard protein concentration of the *E. faecalis* and the active component concentration of the lime extract were measured via the Bradford method (Bio-Rad, Hercules, California, U.S.A.) using Bovine serum albumin (Merck,

Darmstadt, Germany) as the standard protein. Spectrophotometry detected the interaction of *E. faecalis* with lime extract at a wavelength of 595 nm^{13,15}.

The principle of incubation time-based interaction activity on the microplate 96 wells series used in this research based on Gamble's working principle¹⁶; it was modified using violet crystalline and safranin staining. First, 96 wells of the triple microplate series were coated with 50 µl of MHB (Thermo Fisher Scientific Inc., Paisley, UK), settled for 15 minutes, and then aspirated. Second, 50 µL of *E. faecalis* was added and then incubated for 15 minutes at room temperature. Third, 100 µL of the lime extract was added and incubated for 6 hours, 12 hours, 24 hours, 48 hours, and 72 hours (as adapted from research conducted by Bachtiar¹⁷). All of the residues of the test materials (*E. faecalis* + lime extract) in the microplate wells were aspirated and then settled for 10 minutes at room temperature. Then, 50 µL of 2% violet crystalline were added to each well for 5 minutes; the wells were washed with phosphate buffer saline (PBS) two times (Merck, Darmstadt, Germany).

A total of 100 µL of Lugol solution was added for 1 minute and then washed with PBS. The rest of the cell metabolism that was not bacterial cells was dissolved in 96% alcohol for 20 seconds until the dye completely removed. 50 µL of safranin solution was added for 2 minutes and then washed again with PBS¹⁸. The interaction activity between the lime extract and the *E. faecalis* bacteria in the microplate wells was assessed via an Elisa reader using a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 595 nm¹³.

Observation of adhesion mass

The anti-adhesion mass of lime extract against *E. faecalis* that formed on each base of the microplate wells was prepared by adding 100 µl of glycerol for 24 hours to maintain moisture. Visualization was produced by adding 10 µl of immersive oil to each microplate well to be observed under a light microscope at x1000 magnification (Olympus CX21 FS 1, Japan) supported by Optilab Viewer software V 2.2 (Miconus Transdata Nusantara, Jakarta, Indonesia) adapted from a study conducted by Gani¹⁹.

Statistical analysis

E. faecalis acid tolerance and adhesion to lime extract were calculated to determine average values and standard deviations for each concentration. Two-ways analysis of variance (ANOVA) was performed with significance set at $p < 0.05$. The analysis was performed using SPSS ver. 20.0 software.

Results

The experiment was conducted in three replicates. The ANOVA test showed that lime extracts and exposed time gave the significant effect on the pH, optical density of acid tolerance respond of *E. faecalis* in lime extract and optical density of adhesion of interaction activity between *E. faecalis* and lime extract on biofilm ($p < 0.05$). In addition, the interaction between lime extract and exposed time also gave the significant effect on the pH, optical density of acid tolerance respond of *E. faecalis* in lime extract and optical density of adhesion of interaction activity between *E. faecalis* and lime extract on biofilm ($p < 0.05$). The results showed that *E. faecalis* possessed the ability to adapt to

Figure 1. The pH adaptation response of *E. faecalis* to lime extract at different concentration and exposed time.

acidic (pH <7) and alkaline circumstances (pH >7) (Figure 1). Also, *E. faecalis* is tolerant in the acid of lime extract with different intensities at each concentration (Figure 2). Figure 3 shows that the interaction activity of *E. faecalis* in lime extract

influenced by time and concentration were significant ($p < 0.01$) with strong correlation (0.98). The results showed that the interaction activities of *E. faecalis* in Lime extract will decrease from 6, 12, 24, and 72 hours.

Figure 2. The optical density of the acid tolerance response of *E. faecalis* to lime extract at different concentration and exposed time.

Figure 3. Optical density of adhesion of the interaction between *E. faecalis* and lime extract at different concentration and exposed time.

E. faecalis did not express an ability to adapt to the acidic pH of lime extract after interacting with fosfomycin (as a positive control) (Figure 1), although it still expressed acid tolerance response (Figure 2). Its interaction activity was robust (Figure 3). The mass profile also indicated interaction activity between lime extract and *E. faecalis*. Antibiotics are capable of forming a covalent bond to activate the cysteine residue of a bacterial cell, triggering UDP-N-acetylglucosamine to form hydrogen bonds. It inhibits the synthesis of peptidoglycan as an antibacterial defense.

Dataset 1. pH adaptation of *E. Faecalis* in lime extract based on replications

<http://dx.doi.org/10.5256/f1000research.13990.d196643>

Dataset 2. Optical density (OD) of acid tolerance respond of *E. Faecalis* in lime extract based on replications

<http://dx.doi.org/10.5256/f1000research.13990.d196644>

Dataset 3. The OD value of the interaction activity of *E. Faecalis* in lime extract based on replications

<http://dx.doi.org/10.5256/f1000research.13990.d196646>

Discussion

According to Sitanggang *et al.*²⁰, the lime extract has a highly acidic pH ranges (1.7–3.1). The acidity is generated by citric acid and amino acids, while the essential oils contribute to maintaining its acidic pH²¹. Citric acid is reported to play a crucial role as a natural material to maintain pH balance and possesses antibacterial activity²². Figure 1 shows an acidic pH of 2.9 on the extracted bar (without *E. faecalis*). After *E. faecalis* was added at various concentrations, there was a significant change (increase) in the acidic pH of lime extract (as a negative control) ($p < 0.05$). The increased acidic pH of lime extract from 6.25% ($\mu\text{g/ml}$) to 100% ($\mu\text{g/ml}$) (see Figure 1) indicates that *E. faecalis* can adapt to environments with an acidic pH (2.9–4.2) at a temperature of 37°C. Morandi reported that *E. faecalis* could adapt to situations with a low pH and temperature, although they become less sensitive; however, when adjusted to a pH of 5.0 at 25°C, they display increased sensitivity within 10 hours^{23,24}.

E. faecalis had an acid tolerance response to lime extract that significantly increased as the concentration of the lime extract increased (Figure 2) ($p < 0.05$). The increased acid tolerance response correlates with the characteristics of Enterococcal strains producing lipoteichoic acids that contribute to biofilm formation and have resistance to antibacterial agents, including tolerance to acidic environmental change²⁵. Molecularly, the acid tolerance response of *E. faecalis* is influenced by the *EfCitH* gene, which encodes the citrate transporter protein on the surface

of the cell membrane that acts to maintain the balance of the effects of citric acid generated from the environment²⁶. Sarantinopoulos found that enterococcal strains have metabolic potential against the citrate metabolism; this supports their acid tolerance response to environmental influences such as aroma and fermentation products²⁷. In this research, Fosfomycin with a pH of 7.2 (Figure 1) could still slightly tolerate the acidic effects. The acid tolerance response is related to the ability of *E. faecalis* to grow in environments with an alkaline pH (9.5–12) within 48–72 hours¹².

In general, the interaction activity between *E. faecalis* and lime extract at all concentrations was lower than the interaction activity between *E. faecalis* and fosfomycin. Due to its size, the lime extract was considered to be stable based on its treatment concentration (Figure 3). Based on the incubation time, the interaction activity between *E. faecalis* and lime extract at all concentrations within 6–12 hours at the temperature of 37°C was also relatively stable (Figure 3). Varoni *et al.*²⁸ reported that anti-adhesion activity between plant polyphenol-rich extract and *Streptococcus mutans* bacteria was at its maximum within 24 hours, while within 6, 7, and 8 hours, the activity was stable but not yet maximal.

Figure 3 shows there was a significant increase in the average concentration bar ($p > 0.05$), with a relatively high error bar (standard deviation value) within 24–72 hours. It indicates that from 24–72 hours, *E. faecalis* begins to adapt and tolerate the temperature and pH of the environment. The mechanism utilized by bacteria to survive heat and low-pH of the environment operate in many different ways. The most successful means of surviving low-pH stress is the complete avoidance of extremely acidic environments. However, none more critical than the sensing of mild acidification to prevent the potentially lethal consequences of the inappropriate production of potentially antigenic proteins. Bacteria that are forewarned by mild acidification can prepare through the induction of a wide range of protective measures. It can alter the composition of the cell membrane, extrude protons, protect macromolecules, alter metabolic pathways, and generate alkaline²⁹.

The interaction activity between lime extract and *E. faecalis* can be assumed to be the antibacterial activity, because there was a decrease in the interaction activity between *E. faecalis* and the biological components of lime extract within 6–24 hours and again within 48–72 hours. The interaction activity is related to the activity of the active ingredients contained in the lime extract, such as flavonoids (polyethoxylated flavones and flavanones), coumarin, and terpenoids, all of which act as antibacterials^{30,31}.

Conclusion

E. faecalis can adapt to environments with a pH of 2.9–4.3 generated by lime extracts. In addition *E. faecalis* also expressed a tolerance response to the acidic environment. The interaction activity between *E. faecalis* and lime extract become stable

within 6–12 hours at a temperature of 37°C. Therefore, the lime extract can be used to inhibit the *E. faecalis* growth.

Data availability

Dataset 1: pH adaptation of *E. Faecalis* in lime extract based on replications [10.5256/f1000research.13990.d196643](https://doi.org/10.5256/f1000research.13990.d196643)³².

Dataset 2: Optical density (OD) of acid tolerance respond of *E. Faecalis* in lime extract based on replications [10.5256/f1000research.13990.d196644](https://doi.org/10.5256/f1000research.13990.d196644)³³.

Dataset 3: The OD value of the interaction activity of *E. Faecalis* in lime extract based on replications [10.5256/f1000research.13990.d196646](https://doi.org/10.5256/f1000research.13990.d196646)³⁴.

Competing interests

No competing interests were disclosed.

Grant information

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Open Peer Review

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In general, the paper would still benefit from a review by a native English speaker to correct the language.

Additional recommended actions to consider are the following:

Abstract

in Methods (2nd line): instead of "Both materials were analyzed for their adaptation, ..." please use "The microbe was analyzed for its adaptation, ..."

Methods

in Culture of *E. faecalis* bacteria: please rephrase the last sentence "The Accurate of the density ..." to make it more understandable.

in Adaptation to pH assay: first sentence: how do the concentration values, given here in %, compare with units of Figures 1 to 3 where concentration is given in micrograms per milliliter?

Results, Discussion

Figure 1: why is the initial pH value (at 0 hours) about the same at all lime extract concentrations? Are there any known inhibitor (buffering) substances in the lime extract (or in the bacterial culture) that could explain relatively constant initial pH at a range of concentrations?

Figure 2: why is the indicated acid tolerance apparently higher towards increasing lime extract concentration also at 0 hours of exposure?

Figure 3: why is the indicated adhesion level apparently higher towards increasing lime extract concentration also at 0 hours of exposure? Where are the error bars mentioned in the text?

The paper is referring in many places to "interaction activity" between lime extract and *E. faecialis*. However, could the results also involve a concentration-dependent interaction between e.g. citric acid and other antibacterial compounds of the extract?

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 29 Mar 2018

Zaki Mubarak

We have made the revision based on suggestion and comment from reviewers

Competing Interests: No competing interests were disclosed.

Reviewer Report 15 March 2018

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Boy M. Bachtiar

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Introduction

The last paragraph:acid tolerance response and pH adaptation of *E. faecalis* when it interacts with lime extract.

Reviewer's comment: please change the bold word with: when the bacterium grows as biofilm in the presence of lime extract with difference concentration.

Method

- "Both materials were made in vitro to analyze".

Reviewer's comment: please change with: The material and bacterium were prepared

- In lime extract, typo.....into a glass

- Culture of *E. faecalis* bacteria; please discard; "bacteria"

- *E. faecalis* ATCC 29212 taken from glycerol

.....and incubator (Mettler, Germany).

Reviewer's suggestion: discard this part, and please replace with:

One colony of *E. faecalis* bacteria was subsequently re-cultured in 5 ml of Mueller-Hinton Broth (MHB) medium (Thermo Fisher Scientific Inc, Paisley, UK) in anaerobic conditions at a temperature of 37°C for 48 hours. Afterward, the *E. faecalis* grown on the liquid medium was synchronized further with McFarland 0.5 (1×10^8 CFU/ml) (TM50, Dalynn Biological Inc., Calgary, Canada). The Accuracy of the density of McFarland standard (typo: it should be standard) can be checked using a spectrophotometer with an absorbance reading of 0.08 to 0.1 at 625 nm¹¹.

Adaptation to pH assay

A total of 50 ml of lime extracts in several different concentrations (100%, 75%, 50%, 25%, 12.5%, and 6.25%) was placed into different beaker glasses. Then, 5 ml containing 1×10^8 CFU/mL of *E. faecalis* in MHB (1:10) were added to each of the beakers. The initial pH

Reviewer's suggestion: please indicate the initial pH that was measured at the zero h., before incubation. Reviewer's suggestion: Next, bacterium-containing beaker was put into incubator (37°C) for 6 hours, 12, hours, 24 hours, 48 hours, and 72 hours in an anaerobic atmosphere using Anaerogen TM GasPack (Oxoid, Basing stoke UK), at each of these times, the beakers' pH was measured using a pH meter (Thermo Fisher Scientific Inc, Paisley, UK). Various changes in pH from 0 hours to the specified time can be used as an indicator of whether *E. faecalis* has a tolerance response to the acidic environment and can adapt to changing pH.

Acid tolerance assay

- "Here, 96 wells of the triple microplate series were coated with 50 µl of MHB".

Reviewer suggestion: Please change to: Here, 50 µl of MHB was put into microplate in triplicate.

- "the test materials (*E. faecalis* + lime extract) derived.....

Reviewer suggestion: please change to: material tested and *E.*

faecalis....."Each well" change to the microplate was put into incubator for 15 min.

- "Then bio-tolerant activity was measured using the Elisa Reader (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 595 nm". I suggest to discard this part, as it does not have a clear meaning,activity was measure by ELISA reader.....

Adhesion assay

- "The standard protein concentration of the *E. faecalis* and the active component concentration of the lime extract were measured via the Bradford method"

It is not clear, why the authors need to analyze (by measuring) the *E. faecalis*'s proteins (I assume the whole cell proteins). Thus, what does the authors would like to say with "the standard protein concentration of *E. faecalis* and the concentration of active component of the lime extract"? My suggestion is please discard this part.

- "Spectrophotometry detected the interaction of *E. faecalis* with lime extract at a wavelength of 595 nm"

It is rather confusing. Spectrophotometry was used to detect the interaction between the bacterium and the lime extract. Did you use this method to determine the bacterium growth rate? I suggest to discard this spectrometric method. Otherwise, please focus the methods (adaptation to pH, acid tolerance, and adhesion assay) used only to address your hypothesis; "that biofilm formation or bacterial adherence ability contributes to *E. faecalis* survival in biofilm-related environment in the presence of lime extract."

- The principle of incubation time-based interaction activity on the microplate 96 wells series used in this research based on Gamble's working principle. I think you only tested the bacterial adherence capability, not interaction activity between *E. faecalis* and.....?

- It was modified using violet crystalline and safranin staining. First, 96 wells of the triple microplate series were coated with.....

Suggestion: Please change this part: "First, microplate in triplicate wells.Second, 50 μ L of *E. faecalis* in (ADD IN WHAT MEDIUM USED)Third, 100 μ L of different concentration of LE was added and incubated for.....

- "A total of 100 μ L of Lugol solution was added for 1 minute and then washed with PBS. The rest of the cell metabolism that was not bacterial cells was dissolved in 96% alcohol for 20 seconds until the dye completely removed. 50 μ L of safranin solution was added for 2 minutes and then washed again with PBS18. The interaction activity between the lime extract and the *E. faecalis* bacteria in the microplate wells was assessed via an Elisa reader using a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 595 nm"

Suggestion:

1. As you only tested mono-species biofilm (*E. faecalis* is a Gram (+) ve bacterium), please add an explanation, why you used both crystal violet and safranin solution?, and you did it similar to the procedure for Gram staining method.
2. Please discard the word: "The interaction activity between the lime extract and the *E. faecalis* Bacteria". It would better to say: the bacterial adherence was assessed using crystal violet and measured using ELISA reader with optical density of 595 nm.
3. Observation of adhesion mass. This method is not necessary for this study, as you have used crystal violet (CV) assay to measure biofilm mass. CV provides a good measure of biofilm mass, although it cannot measure biofilm viability. This means, CV stains both bacteria cells and the polymer substance (carbohydrate) as part of the extra cellular matrix.

Statistical analysis

.....standard deviations for each concentration.

Reviewer comment: why there are no SD displayed in each graph shown in the figures

Results

-The experiment was conducted in three replicates

Reviewer suggestion:

1. Please changed: the experiment was performed in triplicate wells
2. This study showed that the presence of lime extracts decreased pH, but reduction of low pH did not have a significant effect on the ability of *E. faecalis* to adhere and form biofilm, compared to the control (fosfomycin). All result (crystal violet, adaptation to pH, and acid tolerance assays) are shown in Fig .1-3).
3. Interestingly, the tolerance effect was not influenced by exposure time and the concentration of LE set in this study (Fig. 3), and the correlation between time exposure and LE concentration was positive (However, the authors need to show the regression graph as they said $r^2 = 0.98$). Otherwise, please exclude this part.
4. "the results showed that the interaction activities of *E. faecalis* in Lime extract will decrease from 6, 12, 24, and 72 hours. Reviewer's comment: I am wondering what the authors mean with the interaction activity. I suggest to discard this part.
5. The mass profile also indicated interaction activity between lime extract and *E. faecalis*. Antibiotics are capable of forming a covalent bond to activate the cysteine residue of a bacterial cell, triggering UDP-N-acetylglucosamine to form hydrogen bonds. It inhibits the synthesis of peptidoglycan as an antibacterial defense. Reviewer's comment: Please discard this part, as 1. I cannot see the data (mass profile). 2. Antibiotic are capable.....this is not the result of this study. When necessary please put the sentence in the discussion section.

Discussion

Reviewer's comment: please focus the results you got. Especially, please do not repeat the result, and exclude: "The interaction activity between lime extract and *E. faecalis* can be assumed to be the antibacterial activity, because there was a decrease in the interaction activity between *E. faecalis* and the biological components of lime extract within 6–24 hours and again within 48–72 hours. The interaction activity is related to the activity of the active ingredients contained in the lime extract, such as flavonoids (polyethoxylated flavones and flavanones), coumarin, and terpenoids, all of which act as antibacterials". I suggest to rewrite this partThe survival of *E. faecalis* in the presence of LE.....

Conclusion

E. faecalis can adapt to environments with a pH of 2.9–4.3 generated by lime extracts. In addition *E. faecalis* also expressed a tolerance response to the acidic environment. The interaction activity between *E. faecalis* and lime extract become stable within 6–12 hours at a temperature of 37°C. Therefore, the lime extract can be used to inhibit the *E. faecalis* growth.

Reviewer's comment: Your conclusion should succinctly describe the overall result of the experiment. Please delete: 1. The interaction activity between *E. faecalis* and lime extract become

stable within 6–12 hours at a temperature of 37°C. 2/. Therefore, the lime extract can be used to inhibit the *E. faecalis* growth.

Please refer your conclusion to the title that reflects the result.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral microbiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 29 Mar 2018

Zaki Mubarak

Revision has been made according to the comment of the reviewer

Competing Interests: No competing interests were disclosed.

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