



RESEARCH ARTICLE

REVISED The acid tolerance response and pH adaptation of *Enterococcus faecalis* in extract of lime *Citrus aurantiifolia* from Aceh Indonesia [version 2; referees: 2 approved]

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v2 First published: 07 Mar 2018, 7:287 (doi: 10.12688/f1000research.13990.1)
 Latest published: 11 Apr 2018, 7:287 (doi: 10.12688/f1000research.13990.2)

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. The microbe was analyzed for its pH adaptation, acid tolerance response, and adhesion assay 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 as well as 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* in different concentrations of 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.

Open Peer Review

Referee Status:  

	Invited Referees	
	1	2
REVISED		
version 2	report	
published		
11 Apr 2018		
version 1		
published	report	report
07 Mar 2018		

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Author roles: **Mubarak Z:** Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Resources, Software, Supervision, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; **Soraya C:** Data Curation, Investigation, Project Administration, Resources, Software, Writing – Original Draft Preparation

Competing interests: No competing interests were disclosed.

How to cite this article: Mubarak Z and Soraya C. **The acid tolerance response and pH adaptation of *Enterococcus faecalis* in extract of lime *Citrus aurantiifolia* from Aceh Indonesia [version 2; referees: 2 approved]** *F1000Research* 2018, 7:287 (doi: [10.12688/f1000research.13990.2](https://doi.org/10.12688/f1000research.13990.2))

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Grant information: The author(s) declared that no grants were involved in supporting this work.

First published: 07 Mar 2018, 7:287 (doi: [10.12688/f1000research.13990.1](https://doi.org/10.12688/f1000research.13990.1))

REVISED Amendments from Version 1

In response to all comments and suggestions from the reviewers 1 and 2, we have done some revisions in the new version of our article:

1. Improvement of method section of Abstract.
2. Small changes in the first and last paragraphs of Introduction.
3. Small revision in "Materials" and "Lime extract" sections in Methods; and significant improvement in the "Culture of *E. faecalis*", "Adaptation to pH assay", "Acid tolerance assay" and "Adhesion assay" sections in the Methods. We also deleted the "Observation of adhesion mass" section as recommended by one of the reviewers that this is not relevant to the objectives of our study.
4. We also deleted the term "*E. faecalis* interaction with the lime extract" in all relevant sentences throughout the manuscript because we did not assess this issue.
5. Changes we made in the Results and Conclusion sections are those recommended by reviewer 1.
6. In addition to improvement, reconstruction and enrichment of the Discussion section, we added bar (standard deviation) in our figures, as recommended by both reviewers, and adjusted unit of lime extract concentration for each figure displayed, and added relevant sentences in response to comment given by reviewer 2.
7. As additional information have been included in the Discussion, some new references were added.

See referee reports

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. This bacterium 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 (*Citrus aurantiifolia*). Lime peel extract 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 pneumoniae*, *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 the bacterium grows as biofilm in the presence of lime extract with different concentration.

Methods

Materials

The lime extract and *E. faecalis* (ATCC-29212) were used in this study. The extractions were conducted at the Laboratory of Microbiology at the Faculty of Veterinary, University of Syiah Kuala, Darussalam, Banda Aceh, Indonesia. The material and bacterium were prepared *in vitro* to analyze the pH adaptation, acid tolerance response, and interaction activity of *E. faecalis* in different concentration of 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 a glass container and macerated 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*

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 accurate density of McFarland standard was 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% v/v, 75% v/v, 50% v/v, 25% v/v, 12.5% v/v, and 6.25% v/v) was put 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 each mixture prior to incubation (0 hours) was 2.89, 2.75, 2.91, 2.92, 2.95, 2.98 and 3.10, respectively. 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 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, 50 µl of MHB was put into a microplate in triplicate (Thermo Fisher Scientific Inc., Paisley, UK). The microplate was incubated at room temperature for 15 minutes and vacuumed. The materials tested and *E. faecalis* derived from the incubation processes at 6 h, 12 h, 24 h, 48 h, and 72 hours were added to each well. The microplate was put into an incubator 37 °C for 15 minutes. Then bio-tolerant activity was measured by Elisa Reader (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 595 nm.

Adhesion assay

Adhesion assay was conducted based on the principles of Gram-staining¹⁴. This incubation time-based interaction activity on the microplate 96 wells series was done following the Gamble's working principle¹⁵. It was modified using violet crystalline and safranin staining to confirm the possible occurrence of bacterial contaminants. First, microplate in triplicate wells were coated with 50 µl of MHB (Thermo Fisher Scientific Inc., Paisley, UK) for 15 min, and aspirated. Second, 50 uL of *E. faecalis* in MHB were added and incubated for 15 min at room temperature. Third, 100 uL of different concentrations of LE were added and incubated for 6 h, 12 h, 24 h, 48 h, and 72 hours (as adapted from research conducted by Bachtiar)¹⁶. All residues of the test materials (*E. faecalis* + lime extract) in the microplate were aspirated and the plate was settled for

10 min 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 washed with PBS. Cell metabolites and dye were removed by the addition of 100 µL of 96% alcohol for 20 seconds. Safranin solution, 50 µL, were added for 2 minutes and washed with PBS^{17,18}. The microplate was measured using an Elisa reader using with optical density of 595 nm¹³. For each period, the turbidity of the medium was visually compared with a 0.5 McFarland standard

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 performed in triplicate wells. 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 results (adaptation to pH, acid tolerance assays and interaction activity are shown in Figure 1–Figure 3). Interestingly, the tolerance effect was not influenced by exposure time and the concentrations of lime peel extract set in this study (Figure 3), and the correlation between time exposure and lime extract concentration was positive ($r^2 = 0.98$).

E. faecalis did not express an ability to adapt to result in pH changes after interacting with fosfomycin (as a positive control) (Figure 1), although it still expressed acid tolerance response (Figure 2) and robust adhesion activity (Figure 3).

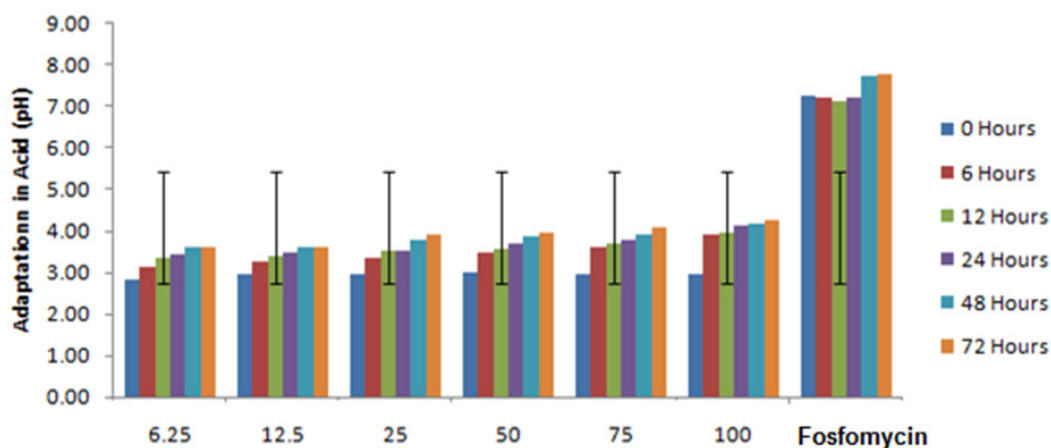


Figure 1. The pH adaptation response of *E. faecalis* to lime extract at different concentration and exposed time.

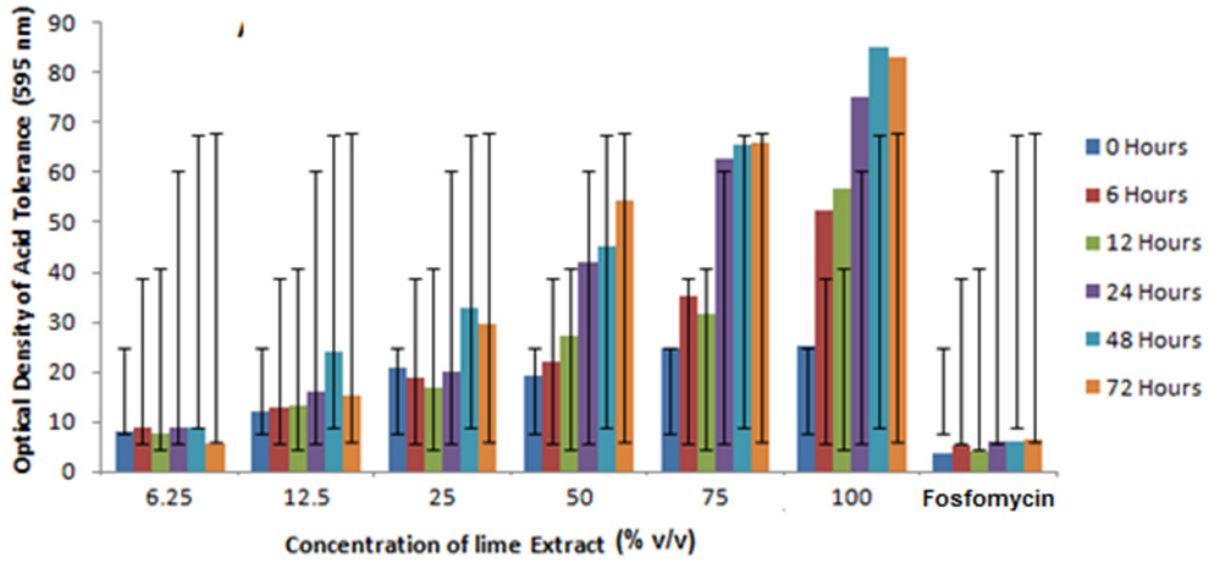


Figure 2. The optical density of the acid tolerance response of *E. faecalis* to lime extract at different concentration and exposed time.

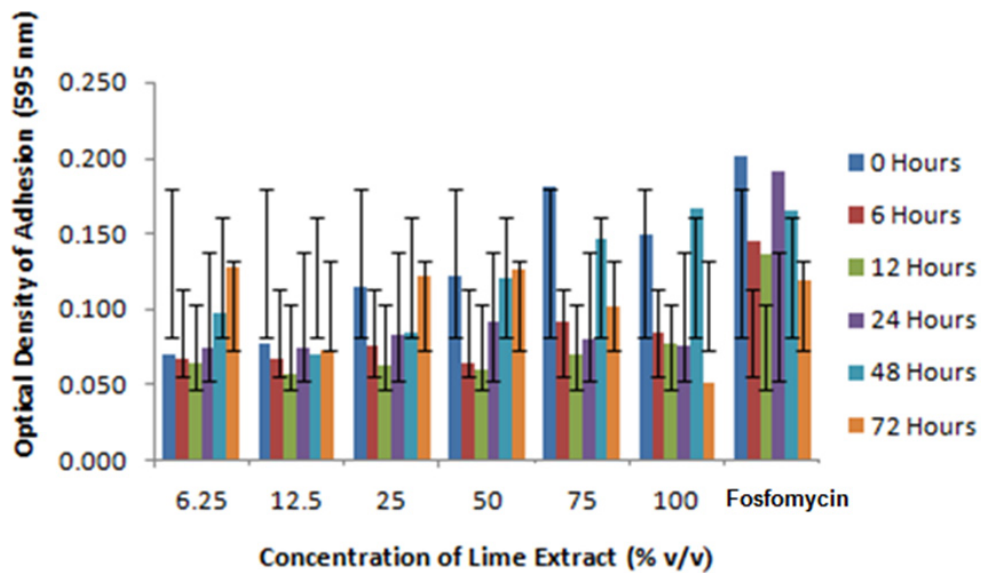


Figure 3. Optical density of adhesion of *E. faecalis* at different concentration of lime extracts and exposed time.

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

In searching for pH adaptation of *E. faecalis* to ethanolic extract of lime peel, the initial pH of concentrated extract (100% v/v)

without *E. faecalis* was 2.89. This is in agreement with those reported by Sitanggang *et al.*¹⁹ that the water extract of lime has highly acidic pH ranges (1.7–3.1). As illustrated in **Figure 1**, this highly acidic pH only slightly changed under serial dilution and after the addition of *E. faecalis* suspension prior to incubation (0 hour).

This relatively stable pH of lime extract after serial dilution in water and after the addition of *E. faecalis* might be related to the presence of buffering compounds in the extract that is able to maintain pH. According to Bolhari *et al.* the juice of lime (*C. aurantiifolia*) contains 88% water, 6–8% citric acid, 2% potassium citrate and calcium, 0.4–0.6% and other substances²⁰. The water extract of the fruit contains a number of bioactive compounds such as alkaloids, phenols, flavonoids, steroids, terpenoids, reducing sugar, saponins and cardiac glycosides²¹. Moreover, the peel of *C. aurantiifolia* contained 7 percent essential oil consisting of 46 compounds, most of which are terpenes²². The acidity is generated by citric acid and amino acids, while the essential oils contribute to maintaining its acidic pH²³. Citric acid is reported to play a crucial role as a natural material to maintain pH balance and possesses antibacterial activity²⁴.

After incubation there was a significant increase in the acidic pH of lime extract containing *E. faecalis* compared to lime extract alone (as a negative control) ($p < 0.05$). The increased pH occurred in all different concentration of lime extracts, from 6.25% (v/v) to 100% (v/v) (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. The ability of *E. faecalis* to adapt to situations with a low pH and temperature has been reported by Morandi *et al.*²⁵ in the experiment using pH adjusted to pH of 5.0 at 25°C within 10 hours.

Whilst better *E. faecalis* growth was observed in different lime extract concentrations with long exposure, increased acid tolerance response shown by the microbe as the concentration of the lime extract increased (**Figure 2**) ($p < 0.05$). These phenomena probably relate to the ability of *E. faecalis* to produce a number of virulence factors in the extreme environment, such as the presence of potent antimicrobial agents or highly acidic condition²⁶. Among them is lipoteichoic acids that contribute to biofilm formation, a bacterial community-based resistant mechanism developed by certain bacteria to survive in extremely disadvantages environment²⁷.

Molecularly, the acid tolerance response of *E. faecalis* is influenced by the *EjCitH* gene, which encodes the citrate transporter protein on the surface of the bacterial cell membrane that acts to maintain the balance of the effects of citric acid generated from the environment²⁸. 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 products²⁹. In this research, in the presence of fosfomycin with a pH of 7.2 (**Figure 1**) *E. faecalis* could still slightly show pH tolerance. The acid tolerance response is related to the ability of certain

E. faecalis strains to grow in environments with an alkaline pH (9.5–12) within 48–72 hours¹².

In general, *E. faecalis* showed progressively decreased adhesion ability in the presence of different concentrations of lime extract within 6 – 24 hours (**Figure 3**). The adhesion started to increase after the microbe was exposure to different concentration of lime extract for 24 hours. The relatively high error bar (standard deviation values) obtained indicates such variation might be existed among the *E. faecalis* isolates toward the pH and concentration of lime extracts.

Varoni *et al.*³⁰ 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. This is probably caused by the adaptation and tolerant mechanisms developed by *E. faecalis* against bioactive compounds presence in the extracts. Lime peel extract contains a number of metabolites (phenols, flavonoids, hydrogen peroxide, tannins, alkaloids, and saponins) have various therapeutic properties such as antibacterial, antioxidant, antifungal, analgesic, and anti-inflammatory⁹.

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 alkaline³¹.

The lower adhesion activity observed in *E. faecalis* exposed to different concentrations of lime extract compared to that exposed to fosfomycin indicated better antibacterial activity of lime extract on the microbe than that of fosfomycin. Among active ingredients contained in the lime extract are flavonoids (polyethoxylated flavones and flavanones), coumarin, and terpenoids, all of which act as antibacterials³². Extract prepared from peel, fruit and leaves of lime show promising antibacterial activity against some microbes belong to both gram positive bacteria (*Staphylococcus aureus*, *S. epidermis* and *E. faecalis*) and gram negative bacteria (*Klebsiella pneumonia* and *Proteus vulgaris*)³³.

Conclusion

Enterococcus 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 of *E. faecalis* in different concentrations of 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)³⁴.

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)³⁵.

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)³⁶.

Competing interests

No competing interests were disclosed.

Grant information

The author(s) declared that no grants were involved in supporting this work.

Acknowledgments

We would like to thank the Laboratory of Microbiology at the Faculty of Veterinary, Syiah Kuala University, Darussalam, Banda Aceh, Indonesia for preparing the *E. faecalis* ATCC 29212 and lime extract as the test materials used in this study.

References

- Stuart CH, Schwartz SA, Beeson TJ, *et al.*: **Enterococcus faecalis: Its role in root canal treatment failure and current concepts in retreatment.** *J Endod.* 2006; **32**(2): 93–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Saber S, El-Hady SA: **Development of an intracanal mature Enterococcus faecalis biofilm and its susceptibility to some antimicrobial intracanal medications; An in vitro study.** *Eur J Dent.* 2012; **6**(1): 43–50.
[PubMed Abstract](#) | [Free Full Text](#)
- Morente EO, Fernández-Fuentes MA, Burgos MJ, *et al.*: **Biocide tolerance in bacteria.** *Int J Food Microbiol.* 2013; **162**(1): 13–25.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Fisher K, Phillips C: **The ecology, epidemiology and virulence of Enterococcus.** *Microbiology.* 2009; **155**(6): 1749–57.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Distel JW, Hatton JF, Gillespie MJ: **Biofilm formation in medicated root canals.** *J Endod.* 2002; **28**(10): 689–93.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Arias-Moliz MT, Ferrer-Luque CM, Espigares-García M, *et al.*: **Enterococcus faecalis biofilms eradication by root canal irrigants.** *J Endod.* 2009; **35**(5): 711–14.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kandaswamy D, Venkateshbabu N, Gogulnath D, *et al.*: **Dentinal tubule disinfection with 2% chlorhexidine gel, propolis, morinda citrifolia juice, 2% povidone iodine, and calcium hydroxide.** *Int Endod J.* 2010; **43**(5): 419–23.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Michalopoulos AS, Livaditis IG, Gougoutas V: **The revival of fosfomycin.** *Int J Infect Dis.* 2011; **15**(11): e732–e39.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Loizzo MR, Tundis R, Bonesi M, *et al.*: **Evaluation of Citrus aurantifolia peel and leaves extracts for their chemical composition, antioxidant and anticholinesterase activities.** *J Sci Food Agric.* 2012; **92**(15): 2960–67.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Nwankwo IU, Ekpe IN, Osaro-Matthew RC: **Synergistic antibacterial potentials of Citrus aurantifolia (lime) and honey against some bacteria isolated from sputum of patients attending Federal Medical Center Umuahia.** *Int J Curr Microbiol Appl Sci.* 2015; **4**(4): 534–44.
[Reference Source](#)
- Maraki S, Samonis G, Rafailidis PI, *et al.*: **Susceptibility of urinary tract bacteria to fosfomycin.** *Antimicrobial Agents Chemother.* 2009; **53**(10): 4508–4510.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- McHugh CP, Zhang P, Michalek S, *et al.*: **pH required to kill Enterococcus faecalis in vitro.** *J Endod.* 2004; **30**(4): 218–19.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Gani BA, Chismirina S, Hayati Z, *et al.*: **The ability of IgY to recognize surface proteins of Streptococcus mutans.** *Majalah Kedokteran Gigi.* 2009; **42**(4): 189–93.
[Publisher Full Text](#)
- Nematollahi A, Decostere A, Pasmans F, *et al.*: **Adhesion of high and low virulence Flavobacterium psychrophilum strains to isolated gill arches of rainbow trout Oncorhynchus mykiss.** *Dis Aquat Organ.* 2003; **55**(2): 101–107.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Gamble R, Muriana PM: **Microplate fluorescence assay for measurement of the ability of strains of Listeria monocytogenes from meat and meat-processing plants to adhere to abiotic surfaces.** *App Environ Microbiol.* 2007; **73**(16): 5235–44.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Bachtiar EW, Bachtiar BM, Dewiyani S, *et al.*: **Enterococcus faecalis with capsule polysaccharides type 2 and biofilm-forming capacity in Indonesians requiring endodontic treatment.** *J Invest Clin Dentistry.* 2015; **6**(3): 197–205.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Soraya CS, Ishaluddin A: **Activity antibacterial of propolis on growth of Streptococcus mutans and Enterococcus faecalis based on in vitro.** *Cakradonya Dent J.* 2011; **2**(3): 332–39.
- Gani BA, Alghassani AQ, Mubarak Z, *et al.*: **Potensi cigarette smoke condensate terhadap peningkatan pembentukan biofilm Candida albicans isolat ATCC 10261. [The potential of cigarette smoke condensate on increase of biofilm formation of Candida albicans ATCC 10261].** *J Syiah Kuala Dent Society.* 2017; **2**(1): 33–39.
[Reference Source](#)
- Sitanggang P, Tambunan E, Wuisan J: **Uji kekerasan Komposit terhadap rendaman buah jeruk nipis Citrus aurantifolia. [Hardness assay on composite in lime extract Citrus aurantifolia immersion].** *e-Gigi.* 2015; **3**(1): 229–34.
[Reference Source](#)
- Bolharo B, Sharifian MR, Aminsobhani M, *et al.*: **Assessing the efficacy of citrus aurantifolia extract on smear layer removal with scanning electron microscope.** *Iranian Endod J.* 2012; **7**(2): 88–97.
[PubMed Abstract](#) | [Free Full Text](#)
- Oikeh EI, Omoregie ES, Oviasogie FE, *et al.*: **Phytochemical, antimicrobial, and antioxidant activities of different citrus juice concentrates.** *Food Sci Nutr.* 2015; **4**(1): 103–109.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Pathan RK, Gali PR, Pathan P, *et al.*: **In vitro Antimicrobial activity of Citrus aurantifolia and its phytochemical screening.** *Asian Pacific J Trop Dis.* 2012; **2**(Supplement 1): S238–S331.
[Publisher Full Text](#)
- Wongkhantee S, Patanapiradej V, Maneenut C, *et al.*: **Effect of acidic food and drinks on surface hardness of enamel, dentine, and tooth-coloured filling materials.** *J Dent.* 2006; **34**(3): 214–20.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Hugenholtz J: **Citrate metabolism in lactic acid bacteria.** *FEMS Microbiol Rev.* 1993; **12**(1–3): 165–78.
[Publisher Full Text](#)
- Morandi S, Brasca M, Alfieri P, *et al.*: **Influence of pH and temperature on the growth of Enterococcus faecium and Enterococcus faecalis.** *Le Lait.* 2005; **85**(3): 181–92.
[Publisher Full Text](#)
- Fabretti F, Theilacker C, Baldassarri L, *et al.*: **Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides.** *Infect and Immun.* 2006; **74**(7): 4164–71.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Sundqvist G, Fildgor D: **Life as an endodontic pathogen: Ecological differences between the untreated and root-filled root canals.** *Endodon Topic.* 2003; **6**: 3–28.
[Publisher Full Text](#)
- Biancato VS, Magni C, Lolkema JS: **Functional characterization and Me ion specificity of a Ca-citrate transporter from Enterococcus faecalis.** *The FEBS J.* 2006; **273**(22): 5121–30.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Sarantinopoulos P, Kalantzopoulos G, Tsakalidou E: **Citrate metabolism by Enterococcus faecalis FAIR-E 229.** *Appl Environ Microbiol.* 2001; **67**(12): 5482–87.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

30. Varoni ME, Lodi G, Sardella A, *et al.*: **Plant polyphenols and oral health: old phytochemicals for new fields.** *Curr Med Chem.* 2012; **19**(11): 1706–20.
[PubMed Abstract](#) | [Publisher Full Text](#)
31. Cotter PD, Hill C: **Surviving the acid test: responses of gram-positive bacteria to low pH.** *Microbiol Mol Biol Rev.* 2003; **67**(3): 429–453.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
32. Sandoval-Montemayor NE, Garcia A, Elizondo-Treviño E, *et al.*: **Chemical composition of hexane extract of *Citrus aurantiifolia* and anti-*Mycobacterium tuberculosis* activity of some of its constituents.** *Molecules.* 2012; **17**(9): 11173–84.
[PubMed Abstract](#) | [Publisher Full Text](#)
33. Al-Rubai LAB, Obaid HH, Saleh HT: **Antimicrobial activity for crude watery extract of seeds of *Citrus aurantiifolia* (lime fruit) against gram positive and negative bacteria *in vitro*.** 2016; 404–416.
[Reference Source](#)
34. Mubarak Z, Soraya C: **Dataset 1 in: The acid tolerance response and pH adaptation of *Enterococcus faecalis* in extract of lime *Citrus aurantiifolia* from Aceh Indonesia.** *F1000Research.* 2018.
[Data Source](#)
35. Mubarak Z, Soraya C: **Dataset 2 in: The acid tolerance response and pH adaptation of *Enterococcus faecalis* in extract of lime *Citrus aurantiifolia* from Aceh Indonesia.** *F1000Research.* 2018.
[Data Source](#)
36. Mubarak Z, Soraya C: **Dataset 3 in: The acid tolerance response and pH adaptation of *Enterococcus faecalis* in extract of lime *Citrus aurantiifolia* from Aceh Indonesia.** *F1000Research.* 2018.
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Current Referee Status:  

Version 2

Referee Report 12 April 2018

doi:[10.5256/f1000research.15751.r33038](https://doi.org/10.5256/f1000research.15751.r33038)



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I have read the revised manuscript, and the authors have made modifications to the manuscript, and have no further comments. Thus, I recommend this work for publication in F1000Research.

Thank you for considering me as a reviewer.

Kindly Regards

Boy M Bachtiar

Competing Interests: No competing interests were disclosed.

Referee Expertise: Oral microbiology

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

Version 1

Referee Report 19 March 2018

doi:[10.5256/f1000research.15206.r31610](https://doi.org/10.5256/f1000research.15206.r31610)



Elza Ibrahim Auerkari 

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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 have read this submission. I 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, Syiah Kuala University, Indonesia

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

Competing Interests: No competing interests were disclosed.

Referee Report 15 March 2018

doi:10.5256/f1000research.15206.r31653



Boy M. Bachtiar

Department of Oral Biology and Oral Science Research Center, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia

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 x 10⁸ CFU/ml) (TM50, Dalynn Biological Inc., Calgary, Canada). The Accuracy of the density of McFarland standart (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 X 10⁸ 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 ul 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 has 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 uL of E. faecalis in (ADD IN WHAT MEDIUM USED)Third, 100 uL of different concentration of LE was added and incubated for....."

- "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 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.

Referee Expertise: Oral microbiology

I have read this submission. I 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, Syiah Kuala University, Indonesia

Revision has been made according to the comment of the reviewer

Competing Interests: No competing interests were disclosed.

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