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The Effect of Nitrite (NO_2^-), Fluoride (F^-) and its combination on *Lactobacillus acidophilus*

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ABSTRACT

Purpose: Dental caries is still main problem in some countries, where bacteria such as *Lactobacillus acidophilus* plays a role in the caries process, then by inhibiting this bacterium could be a way to reduce dental caries. Some researchers investigate the ability of fluoride and nitrite as antibacterial substances separately. Nitrite could collapse the proton gradient of bacteria, while fluoride could inhibit the enolase enzyme. However, there is still limited information regarding the effect of nitrite-fluoride combination on the *Lactobacillus acidophilus*.

Methods: *Lactobacillus acidophilus* ATCC 4356 was grown in the Muller Hinton Agar (MHA) plate together dripped paper disk containing KNO_2 0-3.4%, NaF 0-2.64% and its combination at 37°C in anaerobic condition for 48 hours. Inhibition zone was observed by using caliper. The data was analyzed by using Games Howell test.

Results: Nitrite and fluoride start to inhibit the growth of *Lactobacillus acidophilus* at 0.85% and 0.66% (*p<0.01). Its combination enhances the inhibition zone of *Lactobacillus acidophilus* growth (*p<0.01). Moreover, the combination of KNO_2 0.425%-NaF 0.33%, showed inhibition effect, while KNO_2 or NaF 0.33% only did not show inhibition effect. Furthermore, there is a positive relation between concentration of fluoride, nitrite, its combination with inhibition zone (*p<0.01).

Conclusions: Nitrite and fluoride inhibit the growth of *Lactobacillus acidophilus*, and the nitrite-fluoride combination has a greater effect in inhibiting the growth of *Lactobacillus acidophilus*.

Key-words: *nitrite; fluoride; caries; Lactobacillus acidophilus; human&disease* .

INTRODUCTION

Dental caries is one of the main problems in the oral cavity especially in developing and undeveloping country. This disease gives negative effect to the daily life such as disturbing the mastication, intake nutrition, the growth and development of tooth, including affect to the systemic health, then impact to the quality of life in children nor adults [1]. Database of World Health Organization (WHO) shows that prevalence of dental caries in children around 60-90%, while the other data shows the prevalence of dental caries until 78.3% [2]. Hence the research regarding dental caries is still needed as a consequence of high prevalence.

Dental caries is defined by WHO as demineralized of enamel surface due to acid produced by sugar metabolism of cariogenic bacteria [1,3]. One of the cariogenic bacteria which plays an important role in caries process is *Lactobacillus acidophilus [4–6]*. This bacterium could produce acid through glycolysis which causes lowering the pH and also deminerilize the enamel surface, then dental caries occurred [4,7]. Hence by preventing the growth of cariogenic bacteria such as *Lactobacillus acidophilus* could be one way to prevent the dental caries

Some researchers strive to reveal the potency of several antibacterial agents such as fluoride. This material has been used more than 70 years ago in dental field to reduce the caries rate [8]. Basically, fluoride could prevent the dental caries through couple ways, such as inhibit the growth, enzyme, and acid production of cariogenic bacteria. This fluoride inhibits the growth of pathogenic bacteria by interfere DNA replication, DNA repairing, inhibit the activity of H-ATP-ase, phosphatase, enolase, hexokinase, PEP-PTS, increasing the proton permeability [8–10]. However, the prevalence of dental caries is still high.

Besides fluoride, nitrite (NO_2^-) is also used as antibacterial agent. Recently, the research regarding NO_2^- has been growing rapidly, where this NO_2^- could inhibit the growth of bacteria though several mechanisms, such as collapse the proton gradient, as uncoupled compound, increasing the lipid peroxidase [1,11,12]. Since these materials NO_2^- and fluoride inhibit the bacteria through different mechanisms, hence there is a possibility that combination between NO_2^- and fluoride give better impact in inhibiting the bacterial growth.

MATERIAL AND METHODS

This research is experimental in vitro conducted in Research Centre Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia. The ethical clearance number of this research is 0690/HRECC.FODM/VII/2024 obtained from Universitas Airlangga Faculty of Dental Medicine Health Research Ethical Clearance Commission. *Lactobacillus acidophilus* ATCC 4356 was used in this research.

Bacterial Growing

Lactobacillus acidophilus was cultured in *Brain Heart Infusion Broth* (BHIB) (CM 1135, Oxoid, UK) at 37°C in incubator for 24 hours in anaerobic condition by adding anaerogen (Oxoid™) in anaerobic jar. The bacterial growth suspension was adjusting by adding new BHIB to standardize the turbidity until 0.5 Mc Farland by comparing it to Mc Farland Equivalent Turbidity 0.5 (naked eyes).

The inhibition Effect of Nitrite, Fluoride and Its Combination to Bacteria

The protocol of this research was adapted from Krismariono *et al* with slightly modification [13]. Sterile cotton swab was dipped into the bacterial suspense of *Lactobacillus acidohilus* until wet. Cotton swab was squeezed by pressing on the inner test tube wall, then spread it by scraping evenly on Mueller-Hinton Agar (MHA) (Oxoid, Unitied Kingdom) agar plate. Sterile paper disk was made by using filter paper (Whatman™, pore size 2.5µm), then form a circle shape with diameter 5 mm (autoclave 121°C for 15 minutes). Sterile paper disk was dipped for 1 minutes each in sterile ependorf tube contain KNO_2 (EMSURE®, Germany) 0-3.4%, NaF (EMSURE®, Germany) 0-2.64% and combination of KNO_2 - NaF (KNO_2 0.425% + NaF 0.33%; KNO_2 0.425% + NaF 0.66%; KNO_2 0.85% + NaF 0.33%; KNO_2 0.85% + NaF 0.66%), then incubated anaerobically at 37°C in incubator for 48

hours (Anaerogen, Oxoid™). The determination regarding concentration based on our trial research to achieve the inhibitory threshold. Then, inhibition zone was observed and measured by using a caliper [14]. Four (4) replications for each group were used in this study.

Data Analysis

Normality test was performed by using Shapiro-Wilk test ($p>0.05$), then homogeneity test was performed by using Levene test ($p<0.05$). Data were analyzed by using One-Way ANOVA, Games-Howell test with Statistical Package for the Social Science (SPSS, IBM SPSS 27).

RESULTS

Effect of KNO_2, NaF and its combination on *Lactobacillus acidophilus* growth

Based on this result (table and figure 1), nitrite 0.85% inhibited the growth of *Lactobacillus acidophilus* with inhibition zone 6.812 ± 0.6408 mm. The concentration was in line with inhibition zone, the greater inhibition zone was observed along with increasing the concentration. Nitrite 3.4% gave the highest inhibition zone in table 1 with inhibition zone 12.962 ± 1.0451 mm.

Table and figure 2 showed the inhibition effect of fluoride on *Lactobacillus acidophilus* growth. It showed the fluoride 0.66% could inhibit the growth of *Lactobacillus acidophilus* with inhibition zone 7.3 ± 1.0893 mm. The concentration was in line with inhibition zone, The higher concentration was given, the greater inhibition zone was observed. Moreover, the highest inhibition zone was observed in NaF 2.64% with inhibition zone 13.7375 ± 0.7631 mm.

Combination of KNO_2 0.425% - NaF 0.33% showed inhibition of the growth of *Lactobacillus acidophilus* with inhibition zone 10.1 ± 0.3136 mm (table 3), while KNO_2 0.425% or NaF 0.33% only did not show inhibition effect (table 1-2). Moreover, the combination of those substances showed higher inhibition effect than single KNO_2 or NaF.

Table 1. Inhibition zone of NO_2^- on the growth of *Lactobacillus acidophilus*

No	Groups	Inhibition Zone (Mean ± SD)
1	Aquades	0 mm
2	KNO_2 0.425%	0 mm
3	KNO_2 0.85%	6.81 ± 0.64 mm
4	KNO_2 1.7%	12.01 ± 2.48 mm
5	KNO_2 3.4%	12.96 ± 1.04 mm

Table 2. Inhibition zone of NaF on the growth of *Lactobacillus acidophilus*

No	Groups	Inhibition Zone (Mean ± SD)
1	Aquades	0 mm
2	NaF 0.33%	0 mm
3	NaF 0.66%	7.30 ± 1.09 mm
4	NaF 1.32%	12.30 ± 0.88 mm
5	NaF 2.64%	13.74 ± 0.76 mm

Table 3. Inhibition zone of combination nitrite-fluoride on the growth of *Lactobacillus acidophilus*

No	Groups	Inhibition Zone (Mean ± SD)
1	KNO_2 0.425% - NaF 0.33%	10.10 ± 0.31 mm
2	KNO_2 0.425% - NaF 0.66%	12.80 ± 0.69 mm
3	KNO_2 0.85% - NaF 0.33%	11.62 ± 0.89 mm
4	KNO_2 0.85% - NaF 0.66%	14.05 ± 1.54 mm

Table 4. Significance differences of KNO_2- groups in inhibiting the *Lactobacillus* growth

No	Groups	p-value
1	Aquades – KNO_2 0.85%	0.002*
2	Aquades – KNO_2 1.7%	0.022*
3	Aquades – KNO_2 3.4%	0.001*
4	KNO_2 0.425% – KNO_2 0.85%	0.002*
5	KNO_2 0.425% – KNO_2 1.7%	0.022*
6	KNO_2 0.425% – KNO_2 3.4%	0.001*
7	KNO_2 0.85% – KNO_2 1.7%	0.192
8	KNO_2 0.85% – KNO_2 3.4%	0.003*
9	KNO_2 1.7% – KNO_2 3.4%	0.999

Asterisk symbol* indicating $p<0.01$

Table 5. Significance differences of NaF groups in inhibiting the *Lactobacillus* growth

No	Groups	p-value
1	Aquades – NaF 0.66%	0.008*
2	Aquades – NaF 1.32%	0.001*
3	Aquades – NaF 2.64%	0.000*
4	NaF 0.33% - NaF 0.66%	0.008*
5	NaF 0.33% - NaF 1.32%	0.001*
6	NaF 0.33% - NaF 2.64%	0.000*
7	NaF 0.66% - NaF 1.32%	0.009*

8	NaF 0.66% - NaF 2.64%	0.003*
9	NaF 1.32% - NaF 2.64%	0.504

Asterisk symbol* indicating p<0.01

Table 6. Significance differences between KNO_2^- - NaF and its combination in inhibiting the *Lactobacillus* growth

No	Groups	p-value
1	KNO_2 0.425% - (KNO_2 0.425% - NaF 0.33%)	0.000*
2	KNO_2 0.425% - (KNO_2 0.425% - NaF 0.66%)	0.000*
3	KNO_2 0.85% - (KNO_2 0.85% - NaF 0.33%)	0.004*
4	KNO_2 0.85% - (KNO_2 0.85% - NaF 0.66%)	0.013
5	NaF 0.33% - (KNO_2 0.425% - NaF 0.33%)	0.000*
6	NaF 0.33% - (KNO_2 0.425% - NaF 0.66%)	0.000*
7	NaF 0.33% - (KNO_2 0.85% - NaF 0.33%)	0.001*
8	NaF 0.66% - (KNO_2 0.425% - NaF 0.66%)	0.006*
9	NaF 0.66% - (KNO_2 0.85% - NaF 0.33%)	0.019
10	NaF 0.66% - (KNO_2 0.85% - NaF 0.66%)	0.011

Asterisk symbol* indicating p<0.01

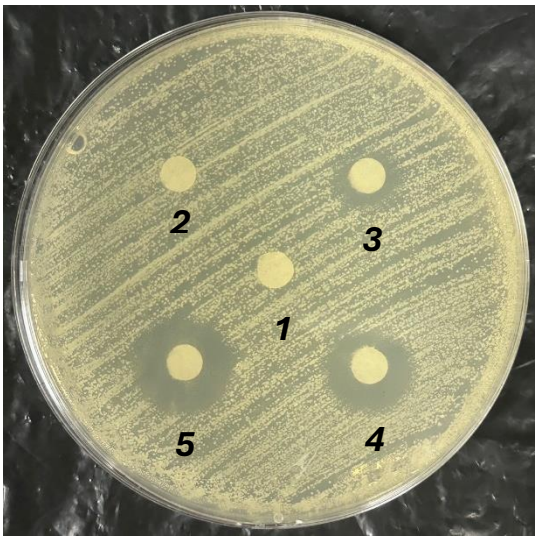

Figure 1. Inhibition zone of Aquades (1); KNO_2 0,425% (2); KNO_2 0,85% (3); KNO_2 1,7% (4); KNO_2 3,4% (5) on *Lactobacillus acidophilus*

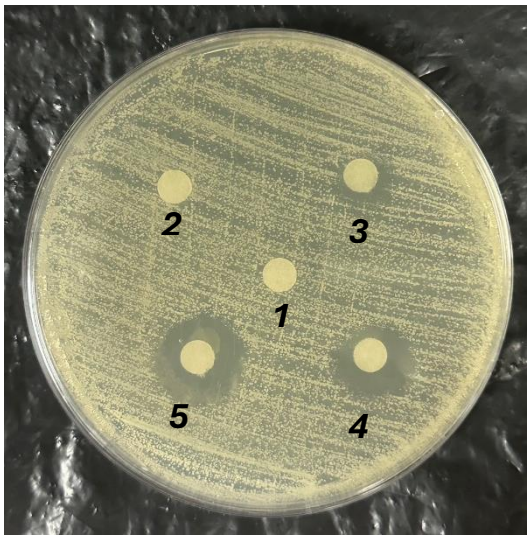

Figure 2. Inhibition zone of Aquades (1); NaF 0.33% (2); NaF 0.66% (3); NaF 1.32% (4); NaF 2.64% (5) on *Lactobacillus acidophilus*

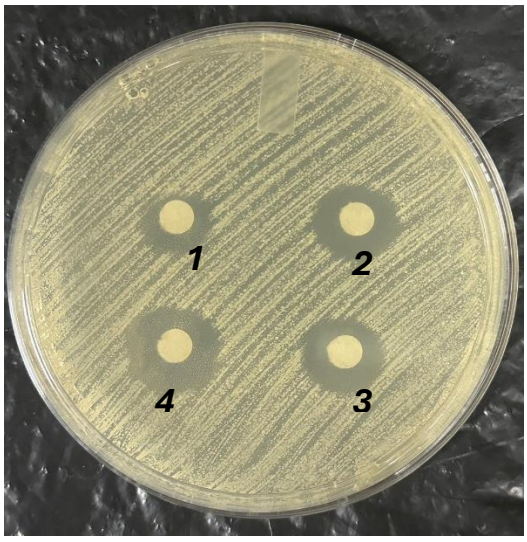

Figure 3. Inhibition zone of combination KNO_2 0,425% - NaF 0.33% (1); KNO_2 0,425% - NaF 0.66% (2); KNO_2 0,85% - NaF 0.33% (3); KNO_2 0,85% - NaF 0.66% (4) on *Lactobacillus acidophilus*

DISCUSSION

Fluoride has been used in a long time ago as antibacterial substances, where it could be used to inhibit the growth of pathogenic bacteria in dental field that play a role in dental disease such as caries [8]. Fluoride could inhibit the growth of

pathogenic bacteria through several mechanisms, such as interfering the replication and repairing DNA, inhibit the activity H-ATPase, phosphatase, enolase, hexokinase, PEP-PTS, increasing the proton permeability [8–10]. Our research result shows fluoride able to inhibit the growth of one pathogenic bacteria, *Lactobacillus acidophilus*. This result is in line with other research, where fluoride varnish 5% could inhibit the growth of *Streptococcus mutans* and *Lactobacillus acidophilus [15]*. Other research also showed that 0.05% sodium fluoride able to inhibit the growth of several pathogenic bacteria such as *Streptococcus mutans*, *Streptococcus sanguinis, Lactobacillus acidophilus [16]*. Our research result also shows the higher the concentration, the greater impact of fluoride to inhibit the growth of *Lactobacillus acidophilus*.

Beside fluoride, nitrite also inhibits the growth of *Lactobacillus acidophilus*, where the higher concentration shows higher impact on inhibition of *Lactobacillus acidophilus* growth. Our result showed KNO_2 at 0.85% inhibits the growth of *Lactobacillus acidophilus* which is observed by inhibition zone. Other researches also showed nitrite could inhibit the growth of pathogenic bacteria such as *Streptococcus* mutans, *Lactobacillus* acidophilus, *Fusobacterium nucleatum* [1,17–19] Our result in line with previous research, where nitrite at 10 mmol/L could inhibit the growth of *Lactobacillus acidophilus* [17] Nitrite could inhibit the growth of pathogenic bacteria through several mechanism such as collapse the proton gradient, inhibit the activity of aldolase, hexokinase, and increasing the peroxidase lipid then it damage the membrane cell [1,20,21]

In this study, we used potassium nitrite (KNO_2) as antibacterial agent due our previous report shows KNO_2 inhibits the growth of cariogenic bacteria [1], while sodium fluoride (NaF) is used, due to many clinicians and references use this agent to prevent the caries process [15,16,22]. However, the combination between these substances is still not establish.

In this research, we would like to know the effect of NO_2^- - fluoride combination, where our result shows combination NO_2^- - fluoride enhancing the inhibition effect than use NO_2^- or fluoride only. Our result also show KNO_2^- 0.425% and NaF 0.33% no effect on inhibition the growth of *Lactobacillus acidophilus,* however combination of those substances (KNO_2^- 0.425% and NaF 0.33%) shows inhibition effect to the growth of *Lactobacillus acidophilus*.

As mention before, the fluoride could inhibit the bacteria through several ways. In order to affect oral bacteria, fluoride either directly inhibits cellular enzymes, increases proton cellular membrane permeability caused by hydrogen fluoride (HF). HF form will enter to the cell, then dissociates in H^+ and F^- in the cytoplasm. The intracellular F^- will inhibit the glycolytic enzyme (enolase) which responsible to convert 2-P-glycerate (2PGA) to Phosphoenolpyruvate (PEP). By inhibiting the production of PEP, then it alters the sugar transport through PEP phophotransferase system. F–also inhibits cell membrane-associated H+-ATPases, reducing the amount of H+ that is excreted from the cell, which decrease the entire glycolytic activity [9,10].

Meanwhile, Nitrite in some references could inhibit the glucose transport (proton dependent-active transport) not through PEP transport system, probably nitrite could act as uncoupler, then causing collapse of the proton gradient across the membrane. Furthermore, nitrite inhibits the aldolase, which responsible as one enzyme in glycolytic pathway. By inhibiting the glycolytic pathway, it impacts in reducing the ATP [1,20,21]. Our result shows nitrite, fluoride could inhibit the growth of *Lactobacillus acidophilus* and combining these two substances give tremendous effect on inhibit the *Lactobacillus* acidophilus. It seems nitrite and fluoride work in different way, then combining substances completes each other, then shows tremendous effect on inhibition the growth of *Lactobacillus acidophilus*.

This research shows the potency of NO_2^- - fluoride combination as the material which is potentially to develop in clinical use such as mouth rinses, tooth paste, irrigation, root canal filling material and others which has relation with antibacterial substances. Further research is needed regarding the analysis of nitrite - fluoride interactions, a more detailed mechanism of nitrite - fluoride in inhibiting bacterial growth, and the ability of the nitrite - fluoride combination to inhibit other pathogenic bacteria to investigate this combination more detail.

CONCLUSIONS

Nitrite and fluoride inhibit the growth of *Lactobacillus acidophilus*, and the nitrite-fluoride combination has a greater effect in inhibiting the growth of *Lactobacillus acidophilus*. Hence, the combination of the two substances is expected to be used in reducing caries prevalence.

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CONFLICT OF INTERESTS

None

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