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The role of inulin extract from mangrove apple (*Sonneratia caseolaris*) and *Lactobacillus plantarum* combination as a synbiotic

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Abstract

Synbiotic yogurt products can be developed using mangrove apple extract inulin as a prebiotic in combination with *Lactobacillus plantarum* to form a probiotic. Therefore, this study aimed to determine the optimal concentrations of inulin extracted from mangrove apple for the viability of *L. plantarum* as a synbiotic in vitro and its antibacterial activity against *Staphylococcus aureus.* The samples were divided into 5 groups and 4 replicates consisting of different concentrations of inulin extracted from mangrove apple (IEMA) at 0, 3, 6, 9, and 12% w/v, which was combined with *L. plantarum* as a synbiotic. The result showed that the addition of IEMA with different concentrations had a significant effect ($p<0.05$) on the viability of *L. plantarum* in gastric juice and bile salt after 5 h of exposure. The total bacteria significantly decreased ($p<0.05$) after 4 weeks of storage period. The antibacterial activity of IEMA at concentrations of 9% and 12% was higher than other treatments as demonstrated by $p<0.05$. IEMA increased the viability with the concentration of 9% being the most effective ($p<0.05$) on acid, bile salt, resistance, and storage periods. Based on the results, IEMA can also inhibit the growth of *S. aureus.*

1. Introduction

Prebiotics serve as a substrate for the growth of bacteria in the colon (Sugiharto, 2016; Kavas *et al*., 2021). Meanwhile, mangrove apple is composed of soluble dietary fiber and inulin, which can be used as a potential prebiotic source (Wibawanti *et al*., 2021). A perfect synbiotic formulation is created by combining prebiotic and probiotic (Jonova *et al*., 2020; Kuo *et al*., 2021). This mixture can be used as a food additive and provide consumers with nutritious products because it does not leave any residue (Sunu *et al*., 2020; Sobotik *et al*., 2021). The synbiotic is a combination of prebiotic and probiotic bacteria (Demirci *et al*., 2017). The prebiotic substrate is converted to short-chain fatty acids (SCFA) by probiotic microorganisms, such as acetic, propionic, and butyric acid. SCFA can lower intestine pH, inhibit pathogenic organisms, and prevent colon cancer (Zubaidah *et al*., 2012). Furthermore, mangrove apple, as a prebiotic candidate, contains polyphenols and other bioactives with antibacterial properties. They have effective antibacterial activity against a variety of Gram-negative and positive bacteria (Thuoc *et al*., 2018).

Probiotics are beneficial microorganisms that has numerous health benefits (Zhao *et al*., 2019; Peng *et al*., 2020). The strains selected for use in the gastrointestinal tract should be safe, viable, and metabolically active. To facilitate colonisation and subsequently benefit the host, ingested probiotic must survive transit through the gastric environment and reach the colon in large quantities (Markowiak *et al*., 2017). The most common microorganism used in probiotics is lactic acid bacteria (LAB) (Sugiarto *et al*., 2015). *Lactobacillus plantarum* is a probiotic bacteria found in the human gastrointestinal tract and has been shown to improve human health (Ola *et al*., 2021). Probiotic bacteria must survive the gastrointestinal tract, tolerate bile, acids, and stomach enzymes, as well as colonise the intestinal epithelium (Lian *et al*., 2003; Ranadheera *et al*., 2012; Duque *et al*., 2021). Currently, *Lactobacillu*s is the most common probiotic bacteria (Shehata *et al*., 2016), but it decreases during storage and in the digestive system (Sensoy *et al*., 2021). Therefore, there is a need to find

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an alternative to improve its viability. Combining probiotic bacteria in synbiotic is one way to boost the ecological functionality and performance. This is because both provide advantages to one another (Sarangi *et al*., 2016).

Synbiotic has been investigated in several studies to increase the number of LAB in the gut. The addition of inulin extracted from chicory roots to low-fat synbiotic yogurt can boost its total LAB (El-Kholy *et al*., 2020). Synbiotic containing prebiotics from gembili tubers and *L. plantarum* improved total LAB and the growth performance in broilers (Setyaningrum *et al*., 2019). The effect of fermented rice bran and *L. plantarum* has been reported to exhibit LAB ability in both the cecum and feces, as well as inhibit pathogenic bacteria (Zubaidah *et al*., 2012). Synbiotic also exhibited antibacterial activity against pathogenic bacteria (Fadare *et al*., 2022). However, studies on IEMA with *L. plantarum* as a synbiotic have not been published. Therefore, this study aims to determine the optimal concentration of inulin extracts from mangrove apple for the viability of *L. plantarum* as a synbiotic *in vitro* and its antibacterial activity against *S. aureus.*

2. Materials and methods

2.1 Preparation of inulin extracted from mangrove apple

The inulin was extracted from mangrove apple using the extraction method described by Wibawanti *et al*. (2021). Mangrove apple was cut into small pieces for extraction, then water with a temperature of 90°C was added. After precipitating with 40% ethanol, the filtrate was stored at -18°C, while the supernatant was eliminated after centrifuging the filtrate for 5 mins at 5000 rpm. IEMA was dried in a cabinet dryer at 50°C for 12 h, then it was sieved in 60 meshes.

2.2 Preparation of cultured Lactobacillus plantarum

Lactobacillus plantarum from LAB was prepared in culture media as described by Jamilah *et al*. (2018). The bacteria (FNCC 0026) were cultured on Man, Rogosa, and Sharp (MRS) agar plates (Merck, Darmstadt, Germany). A colony was selected and inoculated in MRS broth (Oxoid, Hampshire, England) before being incubated for 24 hrs at 37°C under anaerobic conditions to achieve a concentration of at least 10^8 CFU/mL.

2.3 Synbiotic preparation

Synbiotic was prepared based on the mixing of IEMA as prebiotic and *L. plantarum* (Setyaningrum *et al*., 2019). A completely randomized design was used for the experiment. IEMA 0, 3, 6, 9, and 12% (w/v) were used to prepare 5 experimental groups, each with four replicates. Synbiotics were prepared by mixing 10 mL *L. plantarum* namely viable bacterial load of >10^8 CFU/mL and IEMA with different concentrations according to the treatment. They were anaerobically incubated in MRS broth for 24 hrs at 37°C and 4 replicates were used for each treatment.

2.4 The viability of Lactobacillus plantarum in simulated gastric juice

The viability of *L. plantarum* in simulated gastric juice was determined using the method described by Shehata *et al*. (2016). Pepsin (3 g/L; Sigma-Aldrick) was suspended in sterile saline of 0.5% v/v and the pH was adjusted to 2.0 with concentrated 12 N HCl to simulate gastric juices. It was then passed through a membrane to be sterile-filtered. About 1 mL of the synbiotic inulin samples and *L. plantarum* with viable bacterial load of >10^8 CFU/mL was placed in 9 mL MRS broth that has been mixed with simulated gastric juice. The mixture was incubated at 37°C and the number of viable LAB was counted during the incubation period. Furthermore, 1.0 mL samples were suspended in a 1:9 peptone solution (Merck) and serially diluted after mixing the LAB with gastric juice at 0 hr and at predetermined time intervals of 5 hrs. The total number of LAB was then counted on MRS agar and incubated anaerobically for 48 hrs at 37°C. The acid tolerance was determined by comparing the final plate count after 5 hrs to the initial at 0 hr. Four replicates were used for each treatment.

2.5 The viability of Lactobacillus plantarum in bile salt

The modified Lian *et al*. (2003) method was used to test the viability of *L. plantarum* in bile salt. The bile solution was prepared by dissolving 0.5% (w/v) oxgall (Oxoid) in 100 mL distilled water, then the simulations were sterilized for 15 mins at 121°C. About 1 mL of the synbiotic of IEMA and *L. plantarum* with a viable bacterial load of >10^8 CFU/mL were inoculated in 9 mL of MRS broth supplemented with 0.5% bile salt and the pH value was adjusted to 8.0 with 0.1 N NaOH. The mixture was then incubated at 37°C and viable LAB was counted during the incubation period. A total of 1.0 mL samples were suspended in a 1:9 peptone solution (Merck) and serially diluted after mixing the LAB with bile solution at 0 hr and at the predetermined time intervals of 5 hrs. The total number of LAB was then counted on MRS agar and incubated anaerobically at 37°C for 48 hrs. The percentage of the final plate count after 5 hrs compared to the initial at 0 hr was used to determine the bile tolerance and a total of four replicates were used for each treatment.

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2.6 Determination of the total viable count during the storage period

The total number of viable synbiotic bacteria was determined using the spread plate method (Sunu *et al*., 2019). An aliquot (1 mL) of the sample was pipetted into sterile peptone water of 0.1 g/100 mL, while 9 mL for 10^{-1} dilution was made until 10^{-8} dilution was reached. Subsequently, 0.1 mL from each dilution was plated in duplication onto MRS agar (Merck). For 48 hrs, the plate was incubated anaerobically at 37°C. The total viable count was obtained as the logarithms of the number of colony-forming units. The sample was stored at a temperature of 4°C and tested every week for 4 weeks with 3 replicates.

2.7 Determination of antibacterial activity

The disc diffusion method was used to test antibacterial activity as described by Ahmad *et al*. (2018). *Staphylococcus aureus* ATCC 25923 was used as a positive control of bacteria pathogen and the assay began with media preparation. About 15 mL Blood agar media was placed in a sterile petri dish, closed, and cooled to solidify, then 100 µL of the suspension containing 10^8 CFU/mL of the bacteria, was dispensed on the medium using a sterile cotton swab. Subsequently, 100 µL of the IEMA with concentrations of 0, 3, 6, 9, and 12% respectively was dropped on disc paper with a diameter of 13 mm using a micropipette. The media was incubated at 37°C for 24 hrs at the optimum growth temperature.

2.8 Data analysis

All collected data were analysed by SPSS 16 program and the results were obtained using a one-way Analysis of Variance (ANOVA) with a 95% confidence level. The difference between the mean values was assessed with Duncan's Multiple Range Test (DMRT). A value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1 The viability of Lactobacillus plantarum in simulated gastric juice

The addition of IEMA at various concentrations had a significant ($p<0.05$) effect on the total tolerant bacteria at pH 2. Table 1 shows the total bacteria in the synbiotic obtained after using different concentrations of IEMA to simulate gastric juice. After 5 hrs of exposure to simulated gastric juice, the total bacteria in concentrations of 0, 3, 6, 9, and 12% of IEMA were reduced to 1.9, 1.94, 1.89, 1.4, and 1.48 Log (CFU/mL), respectively.

The addition of IEMA at different concentrations culminated in a significant difference ($p<0.05$) in the viability of *L. plantarum* in gastric juice as presented in Figure 1. The viability at concentrations of 9 and 12% with values 85.22±1.71% and 84.65±1.29% were higher ($p<0.05$) than 0, 3, and 6% of IEMA with values of 79.80±2.60%, 79.25±3.64, and 80.10±3.42%, respectively.

Table 1. The total bacteria of *Lactobacillus plantarum* with the addition of IEMA.

Concentration of IEMA	*L. plantarum* count (log CFU/mL) 0 hr	5 hrs
0	9.42±0.27	7.52±0.11^a
3%	9.34±0.33	7.40±0.17^a
6%	9.45±0.34	7.56±0.17^a
9%	9.55±0.18	8.15±0.22^b
12%	9.60±0.27	8.12±0.13^b

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different ($p<0.05$).

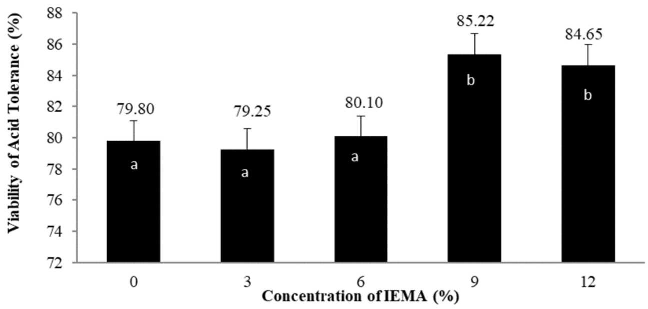

Figure 1. The viability of *Lactobacillus plantarum* in simulated gastric juice. Bars with different alphabets are statistically significantly different ($p<0.05$).

Probiotics must be able to tolerate the low pH of gastric juice in the stomach and bile salt in the gastrointestinal tract (Terpou *et al*., 2019). The total *L. plantarum* in synbiotic with different concentrations of IEMA decreased as the gastric juice during exposure time increased. The addition of IEMA at a higher concentration culminated in a lower reduction in the total number of bacteria in the gastric juice at low pH. *Lactobacillus plantarum* is acid-resistant and can survive at low pH levels. This result was consistent with Ranadheera *et al*. (2012), who examined a comparison of microbial activity throughout gastric juice arrangements, regardless of the carrier food matrix, and revealed that the pH level of the simulated gastric acid used had a significant impact on the viability of probiotics. Sunu *et al*. (2019) demonstrated that enzymes influence low pH LAB growth. The higher the protease enzyme contents of an isolate, the better its resistance to acidic environments. According to Shafi *et al*. (2019), the presence of prebiotics promotes the survival and growth of probiotic cultures in the digestive tract. Based on the study by Grimoud *et al*. (2010), *L. plantarum* can resist various conditions.

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This study showed that the bacteria have tolerance to acidic conditions in the simulation of gastric juice. The viability of *L. plantarum* corresponds with its components inulin from extracted mangrove apple as prebiotic that supports and promotes growth. The prebiotic activity can reach the colon without being digested in the upper gut. It also promotes the growth of one or a few microbes in the gut microbiota. In this study, IEMA served as an energy source and improved LAB survival. Davani-Davari *et al*. (2019) reported that prebiotics can modulate this composition and the function of microorganisms. According to Duque *et al*. (2021), it might be a viable option for promoting probiotic strain growth and improving survival in the gastrointestinal tract. Butt *et al*. (2021) added that LAB can metabolize prebiotics to produce lactate and short-chain fatty acids (SCFAs). Additionally, SCFAs are absorbed through the intestine and used as an energy source, while lactate stimulates gluconeogenesis and SCFAs help lower the pH of the colon.

In this study, the viability of bacteria through the gastrointestinal tract *in vitro* might be due to a combination of prebiotic IEMA and probiotic as synbiotic. Kuo *et al*. (2021) reported that the synbiotic combination of pectin from *L. plantarum* and cacao pod husk might a viable strategy for increasing *L. vannamei* viability. Moreover, the combination of prebiotic and probiotic has a beneficial effect on the digestive system (Arne and Ilgaza, 2021).

3.2 The viability of Lactobacillus plantarum in bile salt

The total *L. plantarum* bacteria in the synbiotic with different concentrations of IEMA was shown in Table 2. There was no significant difference ($p>0.05$) at 0 hr and after 5 hrs of exposure to 0.5% bile solution. The bacteria were reduced by 1.11, 1.07, 1.03, 0.89, and 0.97 Log (CFU/mL) after 5 hrs of 0.5% bile salt exposure in concentrations 0, 3, 6, 9, and 12% of IEMA. The most sensitive condition was observed at 0%, with the bacteria losing 1.11 log CFU/mL. Meanwhile, *L. plantarum* was most tolerant at 9%, declining by only 0.89 log CFU/mL. The total bacteria viability after exposure to bile salt solution was presented in Figure 2.

Table 2. The total bacteria of *Lactobacillus plantarum* in bile salt with the addition of IEMA.

Concentration of IEMA	*L. plantarum* count (log CFU/mL) 0 hr	5 hrs
0	7.38±0.41	6.27±0.51
3%	7.48±0.38	6.41±0.32
6%	7.55±0.36	6.52±0.32
9%	7.50±0.30	6.61±0.29
12%	7.73±0.33	6.76±0.30

Values are presented as mean±SD.

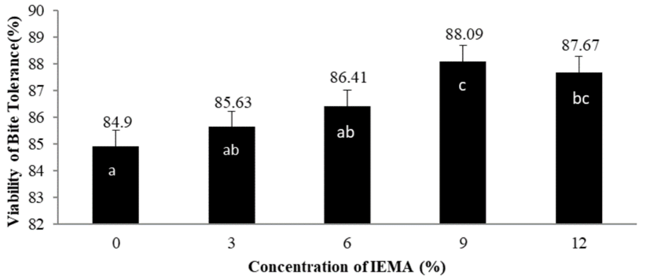

Figure 2. The viability of *Lactobacillus plantarum* in bile salt. Bars with different alphabets are statistically significantly different ($p<0.05$).

The addition of synbiotic with different concentrations of IEMA had a significant effect (p 0.05) on the viability of *L. plantarum*, which was tolerant of simulated intestinal conditions. The viability value of 88.09±1.36% in simulated intestinal conditions with 9% was higher compared to other concentrations namely 0, 3, 6, and 12% with values of 84.9±2.41, 85.63±0.32, 86.41±0.53, and 87.67±1.58%, respectively.

The ability to tolerate bile at 0.5% is required for probiotic bacteria because this concentration is equivalent to that of the physiological bile salt in the duodenum (Puspawati and Arihantana, 2016). The number of bacteria in colonies grown in control versus bile salt treatments was used to calculate resistance observations (Sunu *et al*., 2019). In this study, the total *L. plantarum* in synbiotic with different concentrations of IEMA decreased in the bile salt as the exposure time increased. A higher concentration of IEMA reduced the total number of bacteria with $p>0.05$. The addition of IEMA as a prebiotic and the presence of bile salt might have affected the total bacteria. According to Duque *et al*. (2021), the presence of bile salt and pancreatin affects cell membranes and microorganism viability. Inulin, an oligosaccharide, increased resistance to the bactericidal effects of bile. Patel *et al*. (2004) reported that oligosaccharides from malt, wheat, and barley extract in simulated intestinal conditions improved strains of *Lactobacilli*. In this study, the addition of IEMA decreased the viability as the bile salt exposure time increased. According to Yoha *et al*. (2020), *L. plantarum* in the synbiotic powder simulated *in-vitro* digestion showed a 2-log reduction in viability. The chemical compound of inulin might also have protected probiotic viability during the bile tolerance. Wibawanti *et al*. (2021) reported that IEMA contains a total inulin of 5.08% and it can enhance the growth of probiotics. Wan *et al*. (2020) reported that the bacteria *Lactobacilli* and *Bifidobacteria* perform the fermentation process of inulin in the large intestine and colon. Markowiak and Ślizewska (2013) also found that the colonic mucosa contains SCFAs such as acetate, propionate, and butyrate. They were made from inulin fermentation and provided a significant amount of energy to the host.

3.3 The total viable count of Lactobacillus plantarum during the storage period

The results of the viable count of *L. plantarum* are shown in Figure 3.

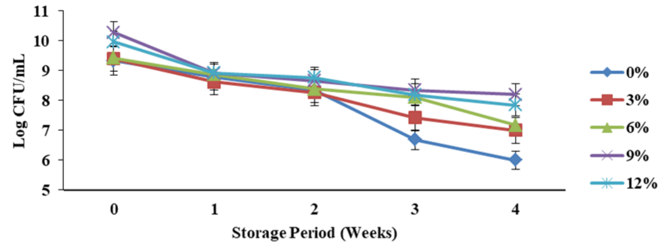

Figure 3. The total viable count of *Lactobacillus plantarum* during storage period.

Table 3 shows that the total viable count of *L. plantarum* with varying concentrations of IEMA in the synbiotic during the storage period at 4°C was significantly different ($p<0.05$). The highest total LAB was found in synbiotic containing 9% IEMA, while the lowest was obtained in the control samples. The higher concentrations of 9 and 12% enhanced the growth significantly compared to other treatments ($p<0.05$). The initial total viable count with the addition of IEMA at 0, 3, 6, 9, and 12% was 9.33±0.60, 9.40±0.21, 9.43±0.23, 10.28±0.62, and 9.96±0.15 log CFU/mL, respectively. Meanwhile, the total viable count at the end of 4 weeks of storage was 6.0±0.10, 7.0±0.04, 7.17±0.12, 8.19±0.35, and 7.85±0.16 log CFU/mL, respectively. Based on the results, the total LAB significantly decreased ($p<0.05$) during the 4 weeks of the storage period. The greatest decline was observed in the control treatment by 3.33 log CFU/mL during 4 weeks of the storage period. The addition of IEMA at concentrations of 9 and 12% significantly reduced the total viable count by 2.09 and 2.11 log CFU/mL ($p<0.05$), while 3 and 6% reduced the number of log CFU/mL by 2.40 and 2.26, respectively.

The results showed a significant interaction ($p<0.05$) between the concentration of IEMA and storage time of 4 weeks on the total amount of *L. plantarum* with a storage temperature of 4°C. This indicates the higher the concentration of IEMA, the higher the total bacteria count. However, storage time has a negative effect on the total number of *L. plantarum*. The higher the concentration of IEMA, the lower the total bacteria count during the storage period.

At the beginning of the storage periods, there was no difference in the viable counts of LAB but as time progressed, significant variations were observed. IEMA was the desired carbon source for LAB, thereby culminating in improved growth and storage viability. The combination of prebiotic from IEMA and probiotic of *L. plantarum* as synbiotic enhanced the viability of LAB.

Sunu *et al*. (2020) found a similar result and stated that prebiotics from garlic extract can provide nutritional support for the growth of *Lactobacillus* bacteria. The results also showed that the viability of *L. plantarum* in the synbiotic with different concentrations of IEMA can survive for 4 weeks. This is consistent with Zhu *et al*. (2020), which found that the probiotic *Lactobacillus sanfranciscens* acts as a probiotic carrier during 4 weeks of storage at 4°C. According to Yoha *et al*. (2020), *L. plantarum* in synbiotic powder with spray freeze drying techniques can survive for 60 days storage period. The results in this study might not only be predicated on IEMA composition but also the storage conditions. Ranadheera *et al*. (2012) found that probiotic functional properties depend on the factors associated with the psycho-chemical compound, manufacturing procedures, ingredients used, and storage conditions.

3.4 The antibacterial activity of synbiotic with the different concentrations of IEMA

The antibacterial activity results of the tested synbiotic containing the different concentrations of IEMA were presented in Figure 4. IEMA was tested for its ability to inhibit the growth of Gram-positive bacteria such as *S. aureus*. Based on the results, the diameter of the inhibition zone tended to increase proportionately to the increasing level of the extract. The lowest activity was found in the control sample without IEMA with a value of 9.57±1.88 mm. The addition of IEMA with concentrations of 9% and 12% culminated in a higher antibacterial activity than other treatments ($p<0.05$) with a diameter zone of 14.77±1.25 mm and 15.48±1.52 mm, respectively. Meanwhile, the concentrations of 3% and 6% did not show a significantly different antibacterial

Table 3. The total bacteria of *Lactobacillus plantarum* in storage periods with the different concentrations of IEMA.

Concentration	Storage periods (weeks)				
of IEMA	0	1	2	3	4
0	9.33±0.60^{b}	8.78±0.11^{cd}	8.34±0.22^{efgh}	6.68±0.42^{l}	6.01±0.10^{m}
3%	9.40±0.21^{b}	8.62±0.08^{cdefg}	8.26±0.05^{efgh}	7.42±0.31^{j}	7.01±0.04^{kl}
6%	9.43±0.23^{b}	8.87±0.02^{c}	8.39±0.05^{defgh}	8.11±0.24^{hi}	7.17±0.12^{jk}
9%	10.28±0.62^{a}	8.91±0.03^{c}	8.66±0.04^{cdef}	8.35±0.20^{defgh}	8.20±0.35^{ghi}
12%	9.96±0.15 ^{a}	8.92±0.07^{c}	8.76±0.19^{cde}	8.19±0.44^{gh}	7.85±0.17^{i}

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different ($p<0.05$).

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activity (p>0.05) with each having the diameter zones of 11.89±0.68 mm and 12.38±0.13 mm. Ampicillin and penicillin were used as positive control with the diameter zone of 29.61±2.24 mm and 33.33±0.30 mm, respectively.

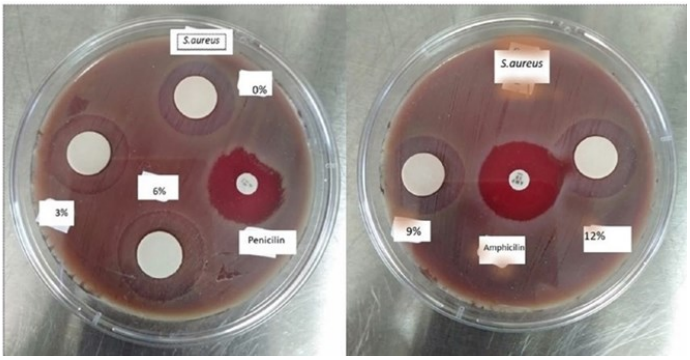

Figure 4. The antibacterial activity of the synbiotic with the different concentrations of IEMA.

The ability of LAB with probiotic activity to inhibit the growth of pathogenic bacteria is another important requirement (Monteagudo-Mera *et al*., 2012). This study used pathogenic *S. aureus* as a Gram-positive bacteria. This was consistent with Ahmad *et al*. (2018), who examined the antimicrobial activity in mangrove apple extract using *S. aureus.* Gram-positive bacteria and *Escherichia coli* as Gram-negative. Furthermore, IEMA contains bioactive compounds with antibacterial activity that can inhibit the growth of *S. aureus*. These effects include an increase in acidic fermentation, which inhibits bacteria growth. The IEMA ability to inhibit the growth of bacteria from different classes might be due to differences in the complexity of cell wall constituents. Thuoc *et al*. (2018) discovered bioactive compounds in mangrove apple, including flavonoids, polyphenols, anthocyanins, antibiotics, antioxidants, and vitamins.

Based on this study, synbiotic was created by combining IEMA and *L. plantarum*. According to Monteagudo-Mera *et al*. (2012), pathogen growth was slowed by LAB's production of bioactive substances such as diacetyl, organic acids, bacteriocins, and hydrogen peroxide, as well as competition for nutrients. Sugiharto *et al*. (2015) also found that antibacterial activity is an important property of probiotic because it prevents pathogenic bacteria growth. Furthermore, Grimoud *et al*. (2019) reported that pathogen inhibition is a primary probiotic criterion, and this process is influenced by the regeneration of gut microbe balance.

4. Conclusion

The combination of IEMA and *L. plantarum* as a prebiotic and probiotic, respectively, has the potential to be used as synbiotic. The 9% extract concentration was the most effective at increasing *L. plantarum* growth, resistance in gastric juice and bile salt, and storage periods. This concentration was also selected due to its ability to inhibit the growth of *S. aureus*. Therefore, future studies should focus on improving synbiotic and digestive conditions in vivo.

Conflict of interest

The authors declare no conflict of interest.

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