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ORIGINAL PAPER

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BALURAN NEEM GUM DRINKING WATER AS A PREBIOTIC CANDIDATE

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ABSTRACT

Background. Neem gum is an exudate from the neem plant (*Azadirachta indica*) with its main constituent being water-soluble non-starch heteropolysaccharide. Gum cannot be degraded in the digestive tract but has the potential to be used as a substrate by lactic acid bacteria. In addition, this material also has the potential to reduce the number of pathogenic bacteria. This ability makes neem gum a candidate for prebiotics, which is becoming the latest food trend.

Materials and methods. Neem gum samples were taken from Baluran and the bacteria used were *Lactobacillus acidophilus* (La) (FNCC 0051), *Bifidobacterium longum* (Bl) (FNCC 0210), *Escherichia coli* (EC) (FNCC 0091), and *Salmonella Typhimurium* (ST) (FNCC 0050). Bacteria were counted by MTT assay and the total plate count method.

Result. The results showed that the number of bacteria exposed to neem gum solution at three different concentrations, namely P5% (w/v), P10% (w/v), and P20% (w/v), experienced an increase in lactic acid bacteria and a decrease in pathogenic bacteria. The increase in the number of lactic acid bacteria was due to fermentation carried out using bacterial extracellular enzymes. The decrease in pathogenic bacteria was due to the inability of the bacteria to utilize heteropolysaccharides as a substrate, which resulted in disruption of the bacterial nutrient uptake. These two things indicate that neem gum solution has the potential to be used as a constituent of prebiotic drinks.

Keywords: prebiotic, neem gum, viability, MTT assay

INTRODUCTION

Neem (*Azadirachta indica*) is a wonder plant from India. Neem is commonly found in Baluran, Indonesia (Prianto et al., 2019). Unfortunately, this plant is often considered to be a nuisance weed. This plant produces abundant gum. Neem gum has not been widely used by local communities in Indonesia. It is a non-starch heteropolysaccharide with a monosaccharide composition, such as L-arabinose, L-fucose, D-galactose, D-glucurionic acid, D-xylose, glucose, and mannose (Moniem et al., 2018). In addition, neem gum also contains other bioactive compounds that act as antibacterial agents, such as NaCl, KCl, salvadourea, salvadorin, saponins, and tannins (Neihaya et al., 2020). The main content of neem gum, which is polysaccharides, has the potential to be used as a functional food ingredient such as a prebiotic. Prebiotics

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are functional food ingredients that cannot be digested or absorbed directly by the body but can be used as a substrate by lactic acid bacteria in the digestive tract. In addition, this material is also able to suppress the growth of pathogenic bacteria to prevent disease (Lestari, 2018).

Two examples of lactic acid bacteria that may be able to ferment neem gum are *Lactobacillus acidophilus* (*L. acidophilus*) and *Bifidobacterium longum* (*B. longum*). Their ability comes from a special enzyme they contain called glycosyl hydrolase, which can hydrolyze complex polysaccharides into various monosaccharides and metabolize them into energy to increase their growth (Husna et al., 2018). The growth of *B. longum* and *L. acidophilus* can suppress the presence of pathogenic bacteria in the gastrointestinal tract because they produce SCFA (short chain fatty acids) (Hardisari and Amaliawati, 2016).

Pathogenic bacteria such as *Escherichia coli* (*E. coli*) and *Salmonella Typhimurium* (*S. Typhimurium*) are those that most often cause digestive tract disorders. Diarrhea cases were recorded in around 4.5 million patients with a mortality rate reaching 4.76% (Kemenkes, 2018). Salmonella itself is reported as the most common cause of foodborne disease. The therapy that is often used in this case is antibiotics such as ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole. Inappropriate use of antibiotics causes multidrug resistance (Eng et al., 2015; Nurjanah, 2020). One form of prevention of this pathogenic disorder is the consumption of prebiotics. Neem gum has potential as a prebiotic candidate because of its main content of polysaccharides and other bioactive compounds that have shown to inhibit the growth of gram-negative pathogenic bacteria in previous studies (Moniem et al., 2018). Based on this description, this study aims to determine the effects of neem gum solution on the number of lactic acid bacteria (*L. acidophilus* and *B. longum*) and enteric pathogenic bacteria (*E. coli* and *S. Typhimurium*) in vitro.

MATERIALS AND METHODS

Materials

The materials used in this study were neem gum crystals purchased from Baluran, Bayuwangi, East Java, *Lactobacillus acidophilus* (La) (FNCC 0051), *Bifidobacterium longum* (Bl) (FNCC 0210), *Escherichia coli* (EC) (FNCC 0091), *Salmonella Typhimurium* (ST) (FNCC 0050), nutrient agar (NA), nutrient broth (NB), MTT (thiazolyl blue tetrazolium bromide) assay kit (reagent and solvent) Biovision brand, inulin (Orafit), HCl, and anaerobic gene.

Bacterial rejuvenation and suspension

Bacterial isolates from the existing stock were grown back in a petri dish containing an agar medium. Then the bacteria were incubated for 24 hours at 37°C anaerobically. After incubation, the bacteria were taken in one ose and suspended in a broth medium and incubated under the same conditions as before. The bacterial suspension was diluted to a concentration of 1×10^8 CFU/ml or 0.5 McFarland. In MTT assay, the bacteria should first be adapted in a well plate (Grela et al., 2015; Ishiki et al., 2018)

Production of neem gum solution

Neem gum crystals were washed thoroughly and dried in the sun to prevent sticking. The neem gum crystals were ground to a powder and filtered using a sieve. The neem gum solution was made by mixing neem gum powder with a nutrient broth medium that had been dissolved in aquadest at 60°C to dissolve easily (Kamaraj, 2018). The concentration of the neem gum solution was made to 5% (w/v), 10% (w/v), and 20% (w/v) in a 1ml nutrient broth medium which had been prepared and homogenized. Prior to use, the solution was sterilized using an auto clave at 121°C for 30 minutes (Bajury et al., 2018).

Inoculation and viability calculation of bacteria using the MTT assay method

The bacterial suspension was inoculated into the well plate up to 50 µl. The bacteria were left for 24 hours to adapt to the conditions. After that, 50 µl of neem gum solution was put into the wells that contained bacteria and incubated for 24 hours (Aristyawan et al., 2016). Incubation using this method was carried out anaerobically at 37°C (Sun et al., 2016; Hegyi et al., 2012). After incubation, 50 µl of MTT reagent was added to each well and then they were incubated again for three hours for the reduction reaction to occur (Noopan, 2019). After a color change from yellow to purple was observed, the formazan crystal was dissolved using 150 µl of MTT solvent.

The microplate was covered with aluminum foil and shaken in an incubator for 15 minutes. After that, the absorbance was read colorimetrically using an ELISA reader with an OD value of 595nm (Hegyi et al., 2012; Benov, 2019). The absorbance obtained was entered into the viability formula as follows (Wati et al., 2016):

% cell viability = ((absorbance of the treatment – absorbance of the medium) / (absorbance of the control cells – absorbance of the medium)) × 100

where:

% cell viability – percentage of live cells
treatment absorbance – optical density (OD) value of the Formazan of each sample after treatment
media absorbance – value of the Formazan control medium
control cell absorbance – OD value of the Formazan on mean control cell.

Bacterial inoculation and calculation using the colony count method

The bacteria that were exposed to the neem gum solution in a test tube were taken in amounts of 0.1ml and poured into a petri dish. The agar medium was added and then shaken slowly to homogenize it. The test medium was incubated in an anaerobic jar at 37°C for 24 hours anaerobically. After 24 hours, the number of bacterial colonies in each plate was counted (Herawati et al., 2018)

RESULTS AND DISCUSSION

The results of bacterial viability using the MTT assay method

Bacterial viability was obtained by entering the absorbance value of the bacteria into the viability formula in the MTT assay method. The control cells had the largest absorbance values, which indicates a large number of live bacteria as well. The viability reached 100% in all four bacteria (see Table 1). The viability of *B. longum* and *L. acidophilus* bacteria in the positive control (K+) that was exposed to inulin solution was 314.5% and 229.60%, respectively. The viability of *B. longum* bacteria in the neem gum treatment group from P5%, P10%, and P 20% were 219.5%, 328.7%, and 448.3%, respectively. The viability values of *L. acidophilus* in the neem gum treatment group were P5% (189.00%), P10% (232.80%), and P20% (242.40%). The viability of these two bacteria exhibited an increasing trend (see Fig. 1).

Viability in the pathogenic bacteria was obtained in the same way. The viability of *E. coli* bacteria in the positive controls (K+) exposed to inulin solution was 66.81%. The viability of *E. coli* bacteria in the treatment group P5%, P10%, and P20% were 75.23%, 62.64%, and 50.15%, respectively. *S. Typhimurium* bacteria produced a positive control (K+) viability of 69.58%. The viability of the *S. Typhimurium* treatment group P5%, P10%, and P20% were 80.73%, 64.66%, and 52.45%, respectively. The hitogram of the viability

Table 1. Average values of absorbance and bacterial viability

No	Groups	Average value of absorbance				Viability (%)			
		ST	EC	La	Bl	ST	EC	La	Bl
1	K–	1.011	0.275	0.216	0.2362	100	100	100	100
2	K+	0.729	0.211	0.378	0.546	69.58	66.81	229.60	314.5
3	P5%	0.833	0.227	0.328	0.4088	80.73	75.23	189.00	219.5
4	P10%	0.684	0.203	0.382	0.5664	64.66	62.64	232.80	328.7
5	P20%	0.571	0.179	0.394	0.7392	52.45	50.15	242.40	448.3

ST – *Salmonella Typhimurium*, EC – *Escherichia coli*, La – *L. acidophilus*, Bl – *B. longum*, K+ = positive control sample (10% commercial prebiotic – inulin), K– = negative control sample (only pure broth media), P5% = 5% neem gum solution, P10% = 10% neem gum solution, P20% = 20% neem gum solution.

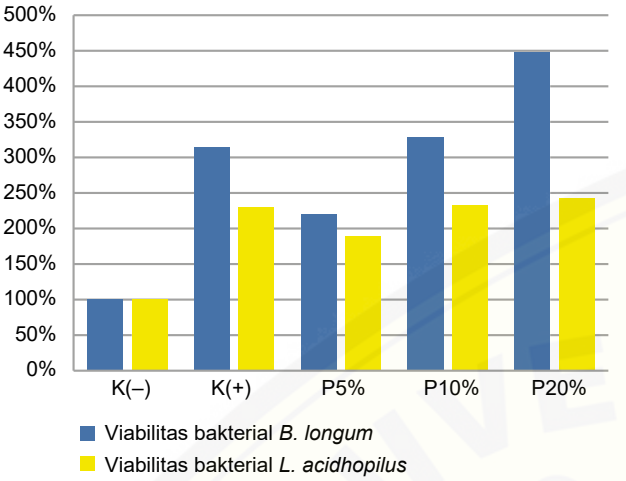

Fig. 1. Viability of lactic acid bacteria

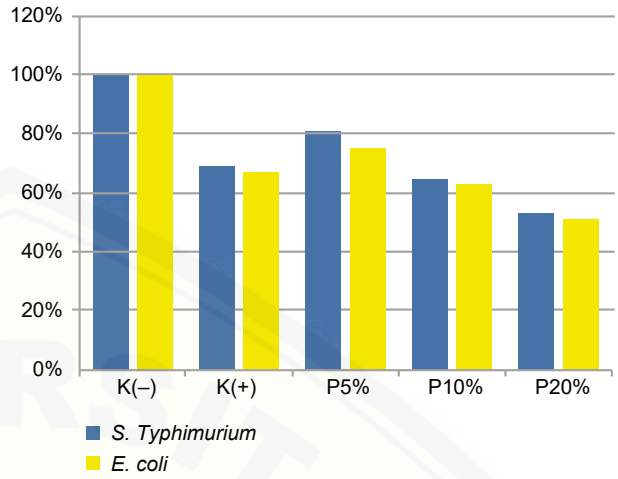

Fig. 2. Viability of pathogenic bacteria

of *S. Typhimurium* and *E. coli* bacteria showed a decreasing trend, while *L. acidopillus* and *B. longum* had an increasing trend (see Fig. 2).

The result of colony counting

The colonies formed on the agar media in the petri dishes were counted as a whole (see Table 2). The results of the study on the exposure of *B. longum* and *L. acidophilus* bacteria to neem gum solution in vitro using the colony count method showed that the negative control (K–) resulted in large bacterial colonies which numbered above 300. Positive control (K+) also resulted in the growth of colonies whose size was above 300. P5% and P10 % in both bacteria showed colonies whose size was above 300. However, there was a decrease in the growth of the two bacteria in the P20% group, namely 119 colonies for *B. longum* bacteria and 109 colonies for *L. acidophilus* bacteria. Then, for the results of the study on the exposure of pathogenic bacteria *E. coli* and *S. Typhimurium* to neem gum solution in vitro using the colony count method, a decrease in the number of colonies was found on exposure to a neem gum solutions with concentrations of 10% and 20%. Negative control (K–) resulted in large bacterial colonies above 300. Positive control (K+) also resulted in the growth of colonies whose size was above 300. P5% in both bacteria did not show any reduction in the number of colonies. The number was still more than 300 colonies. P10% in *E. coli* bacteria was able to reduce bacterial growth to below 300 colonies.

Table 2. Average number of bacterial colonies

Groups	Number of bacterial colonies			
	Escherichia coli	*Salmonella Typhimurium*	*L. acidophilus*	*B. longum*
K– (negative control)	>300	>300	>300	>300
K+ (positive control)	>300	>300	>300	>300
P5% (5% neem gum sol)	>300	>300	>300	>300
P10% (10% neem gum sol)	294	>300	>300	>300
P20% (20% neem gum sol)	194	119	109	119

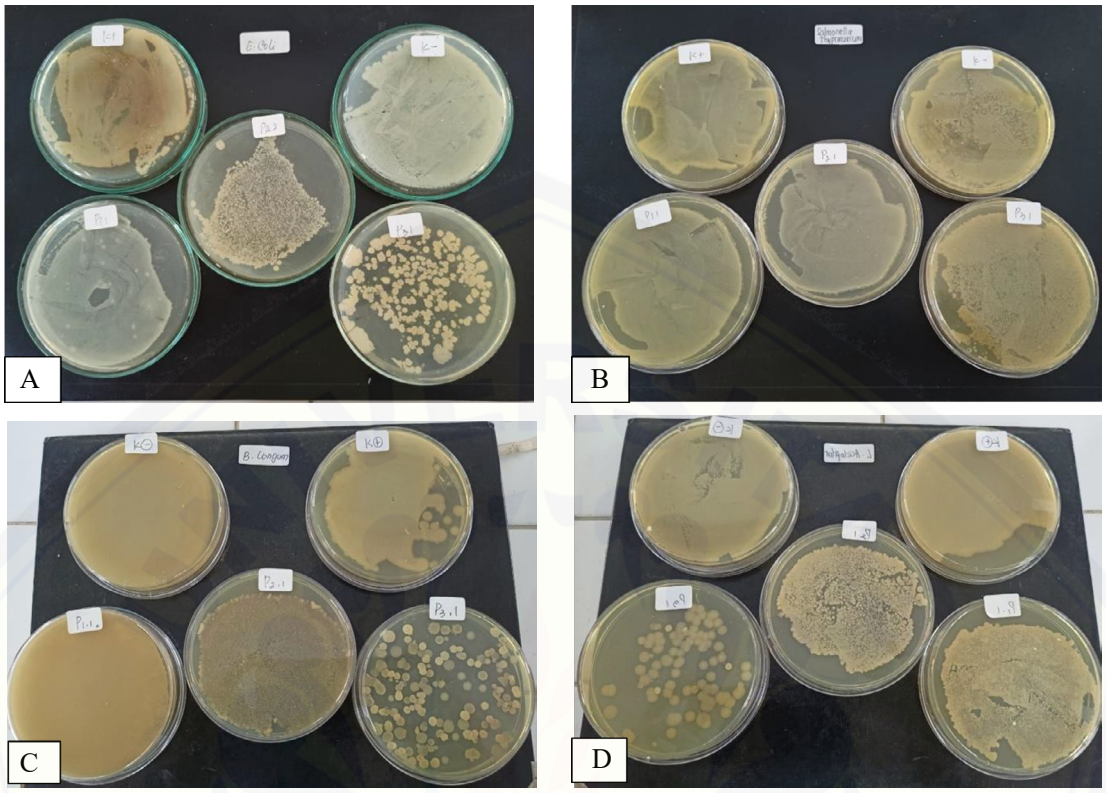

Fig. 3. Bacterial colonies on agar media. A. Colony of *E. coli* bacteria, B. Colony of *S. Typhimurium* bacteria, C. Colony of *B. longum* bacteria, D. Colony of *L. acidophillus* bacteria

While *S. Typhimurium* colonies did not decrease at a concentration of 10%, neem gum solution was able to reduce its growth at the highest concentration of 20%. The four bacterial colonies can be seen in Figure 3.

Data analysis

The data was tested using the IBM SPSS Statistical Data Editor application. Differences between the two groups were tested using the Mann-Whitney test. Data analysis for *B. longum* bacteria showed that there were significant differences the K- with all treatments. The K+ group to P5%, and P20%% and P20% group treatments were significant different, and the P5%, P10%, and P20% also significant different too. The different test for *L. Acidophulus* bacteria the K– group is significant different from the K+, and the K– is different from all treatment groups. While K+ did not differ from other treatments. Further explanation will be presented in the discussion section.

Data analysis for E. Coli bacteria showed that there were significant differences the K– with all treatments. The K+ group to P20%, and P5% and P20% group have significant different too. The same thing happened to the bacteria *S. Typhimurium*. There were also significant differences between the K– and K+ groups, the K– and all the treatment groups, and the P5% and the P20%. Further explanation will be presented in the discussion section.

Discussion

Based on the MTT assay, lactic acid bacteria and pathogenic bacteria have different viability values. The viability value of lactic acid bacteria with neem gum solution was greater than the negative control, while the pathogenic bacteria showed less viability than the negative control. The negative control was used as a benchmark for the research group because these bacteria were not treated and were grown on a culture medium

only. The negative control/cell control results indicate that the inoculated bacteria were alive as a whole.

The positive control in this study used a commercial inulin solution (Orafit). Inulin is a water-soluble complex polysaccharide. In 2016, Shoaib et al. stated that inulin contains complex carbohydrates in the form of fructooligosaccharides which cannot be digested by the enzymes of the digestive system but can be used as nutrients by *B. longum* and *L. acidophilus* bacteria using the fructofuranosidase enzyme. Inulin is also unable to be fermented by either pathogenic bacteria. This was evidenced in this study by an increase in the number of lactic acid bacteria decreasing the number of pathogenic bacteria.

Neem gum solution against lactic acid bacteria

All groups of lactic acid bacteria exposed to neem gum solution produced higher viability than the negative controls. Adding 10% and 20% neem gum solutions also resulted in higher viability of *B. longum* and *L. acidophilus* bacteria compared to the inulin solution. These two bacteria utilize the heteropolysaccharide substrate optimally with the following enzymes: glucosidase, fucosidase, arabinofuranosidase, arabinofuranosidase, galactosidase, mannosidase, and xylosidase (Purwandani et al., 2018; Zabel et al., 2020; Kelly et al., 2021; Wardani et al., 2017).

The viability of the two pathogenic bacteria treated with neem gum solution had a tendency to decrease as the concentration of the solution increased (see Fig. 2). The lowest bacterial viability was found under exposure to 20% neem gum solution. Both of these bacteria, *E. coli* and *S. Typhimurium,* are gram-negative species. These results are also supported by Samrot (2020), who stated that the polysaccharide content contained in neem gum effectively inhibits the growth of gram-negative bacteria, namely *Escherichia coli*, at a maximum concentration of 20%. The effect obtained in the *S. Typhimurium* bacteria showed almost the same results.

The results of the neem gum solution test on lactic acid bacteria with the colony count method showed that the negative control, positive control, 5% neem gum solution, and 10% neem gum solution all had the same number of bacteria (see Table 2). This method cannot be used to determine the exact number of bacteria if the colony is larger than 300. However, the large number of positive controls and neem gum concentrations of 5% or 10% indicates that *B. longum* and *L. acidophilus* can use neem gum solution and inulin solution as a substrate. The occurrence of a decrease in the number of colonies in 20% neem gum solution could have occurred due to the low pH, which dropped as low as 4. *B. longum* and *L. acidophilus* bacteria can grow optimally at pH 6–7 according to the pH in the colon. According to research conducted by Wardani et al. in 2017, a decrease in pH reduces the activity of bacterial growth which is characterized by a decrease in the number of both bacteria.

The difference in the results of a colony count and MTT assay can be caused by several things. The colony count method can misinterpret the shape of the colony as a collection of bacterial cells that is considered a single colony, so the results of the calculations do not show the actual number of bacteria (Soesetyaningsih and Azizah, 2020). This method also takes a long time to prepare and calculate the number of colonies (Rosmania and Yanti, 2020). It is different with the MTT assay, which has a more sensitive, more accurate, and higher sensitivity calculation. Dead bacteria cannot affect the reading process. In addition, the MTT test can easily test more samples at one time, so it can be cheaper and more efficient (Mahfur, 2016; Requena, 2019).

Both tests showed the ability of the two lactic acid bacteria to utilize neem gum solution as a substrate to increase their number. The neem gum polysaccharide is hydrolyzed by breaking the glycosidic bond using the lactic bacteria enzyme. The metabolism of neem gum by *B. longum* and *L. acidophilus* bacteria produces SCFA (short chain fatty acid) compounds. In addition, this metabolism also produces ATP, which is used by these bacteria as energy to grow more (Kelly et al., 2021).

The test results of the colony count method of neem gum solution against these two pathogenic bacteria showed results that supported the MTT assay method. The negative control showed a large number of colonies because the bacteria were only cultured on normal media without treatment. The positive control results also showed a large value. This may be because the dose of the positive control was not sufficient to inhibit this bacterial colony. Different reactions were shown by these two bacteria. Colonies of *E. coli* bacteria began to decrease when exposed to 10% neem

gum solution, while *S. Typhimurium* colony numbers decreased at a solution concentration of 20%. Different bacterial species make this difference possible.

Both methods showed that the neem gum solution was able to reduce bacterial viability and colonies smaller than the negative control. The ability of neem gum solution to reduce the number of enteric pathogenic bacteria is possible due to several factors. The first factor is that these bacteria are unable to utilize the substrate contained in the neem gum solution. The ability of bacteria to ferment substrates in the form of polysaccharides requires the presence of extracellular enzymes that are able to break the glycan bonds between the monosaccharide compositions. Such an enzyme is not contained within these pathogenic bacteria. In 2018, Utama stated that this enzyme can be galactosidase, arabinose, pectinase, glucosidase, or cellulase which are contained in lactic acid bacteria such as *Bifidobacterium longum* and *Lactobacillus acidophilus*. This factor causes disruption in the growth of pathogenic bacteria by blocking their nutritional needs.

The fact that *S. Typhimurium* is not able to ferment the non-starch polysaccharide content of neem gum is also supported by several studies. Neem gum has the largest monosaccharide composition in the form of galactose and arabinose (Moniem et al., 2018). Besides being unfermented, galactose is also known to have the ability to inhibit the growth of pathogenic bacteria (Sari et al., 2020). In 2012, Park stated that *S. Typhimurium* was not able to ferment arabinose. Overall, non-starch polysaccharides in the gastrointestinal tract serve as bacterial attachment agents. Pathogenic bacteria that do not utilize the carbon in the neem gum, make it sticks to the substrate and is then removed along with the feces (de Figueiredo et al., 2020). Other studies have also proven that heteropolysaccharides can damage the structure of the cell walls and membranes of pathogenic bacteria. Polysaccharides work by penetrating the bacterial cell membrane and damaging its integrity (Zhang et al., 2020; Wang et al., 2019). Non-starch polysaccharides also work to break down bacterial cell DNA into small fragments. Abnormality of DNA damages cell life (Rjeibi et al., 2020).

Neem gum contains other active compounds such as NaCl, KCl, salvadora, salvadorin, saponins, and tannins (Neihaya et al., 2020). The elemental chloride in NaCl and KCl causes bacteria to be killed in acidic conditions. Salvadora and salvadorin are the compounds found in the stem of the miswak. These two active compounds have been shown to inhibit bacterial growth (Darout, 2015; Nadir et al., 2020). Saponins cause bacterial cell lysis by increasing the permeability of the bacterial cell membrane. Meanwhile, tannins are able to coagulate the protoplasm of bacteria so that their growth becomes inhibited (Halimah et al., 2019). Phenol is also contained in neem gum. Phenol can damage bacterial membranes and denature cell proteins, which are important systems in bacterial cells (Septiani et al., 2020; Marfuah et al., 2018). A low pH also affects the life of pathogenic bacteria. Neem gum solution has an acidic pH of 4.2–4.8 (Moniem et al., 2018). The higher the concentration of the solution, the lower the pH, causing bacterial cell lysis.

EXPLANATION OF DATA ANALYSIS

Data analysis for lactic acid bacteria

Analysis of the research data was carried out to find the differences between the study groups. The results of the difference test between the negative control group and the positive control group showed a significant difference. This difference was caused by the bacteria in the positive control being exposed to inulin solution, while the negative control had no exposure. The solution in inulin contains more nutrients for the growth of *B. longum* and *L. acidophilus* bacteria so that their viability increases. The difference test between the negative control group and the treatment group also had significant differences. The three concentrations of neem gum solution contained polysaccharide compounds that could be digested by *B. longum* and *L. acidophilus* and used as nutrients to grow more.

Significant differences were also found between the 5%, 10%, and 20% neem gum solution groups. The three concentrations of neem gum solution had significant differences. The concentration is directly proportional to the content of neem gum compounds. Hence, the effect of neem gum solution in increasing bacterial viability is greater at higher concentrations. Between the treatment and positive control groups, there were also significant differences in *B. longum* bacteria. The 5% and 20% neem gum solutions had significant differences with the positive control group, except for

the 10% neem gum solution. These two groups had no significant differences because the viability of *B. longum* bacteria isn't much different, meaning that the 10% neem gum solution and the positive control had the same ability to increase the viability of *B. longum* bacteria. The significant difference between 5% and 20% neem gum solutions and the positive control occurred because of the difference in the percentage of polysaccharides in these groups. The greater the concentration of polysaccharides, the greater the viability of the bacteria obtained. With the difference in concentration between the groups, the ability to increase the viability of *B. longum* bacteria will also be significantly different.

Data analysis for pathogenic bacteria

The results of the difference test between the negative control group and the positive control group showed a significant difference between the two bacteria. This difference was caused by the bacteria in the positive control group being exposed to inulin solution, while the negative control group had no exposure. Inulin is a commercial prebiotic product that has been widely used. In 2019, Farias stated that the structure of the compound inulin is a complex carbohydrate in the form of fructooligosaccharides. These carbohydrates are also strung together in glycan bonds which cannot be broken down by these pathogenic bacteria.

The difference test in the negative control group with all treatment groups resulted in significant differences between the bacteria *E. coli* and *S. Typhimurium*. The negative control group were bacteria that were only cultured on culture media, while the treatment group was given neem gum solution with three different concentrations. The three solutions produced significant differences with the negative control. Neem gum solution contains various complex and bioactive polysaccharide compounds that can reduce the viability of *E. coli* and *S. Typhimurium* bacteria. This content becomes a substrate that cannot be fermented, interferes with the uptake of bacterial nutrients, lowers the pH of the environment, damages the integrity of the cell walls and membranes, increases the permeability of the cell membranes, damages DNA, and coagulates the protoplasm of bacterial cells. Therefore, the viability of pathogenic bacteria exposed to neem gum solution was lower than the negative control.

The difference test between the positive control group and the treatment group showed a significant difference in *E. coli* bacteria at a concentration of 20%, while in *S. Typhimurium* there was no significant difference. Significant differences in *E. coli* can be caused by different bacterial species so that bacteria are more sensitive to the content in the neem gum solution. At the highest concentration, it can be said that the viability of *E. coli* decreased by half or reached the median lethal dose. There was no difference because both of the solutions are made up of non-starch complex carbohydrates that are soluble in water and cannot be degraded by the digestive tract. In addition, *E. coli* and *S. Typhimurium* bacteria were also unable to utilize these two materials as substrates. This situation causes disruption to the life of these bacteria, which ultimately reduces their viability.

Significant differences were also found between the P5% and P20% groups in both pathogenic bacteria. The concentration had a different effect on the bacterial viability seen in P5% and P20%. The concentration is directly proportional to the content of neem gum carried. The effect of neem gum solution in reducing viability is greater at high concentrations. The results showed that the highest concentration resulted in the lowest viability in pathogenic bacteria.

The results of this study indicate that neem gum solution has the potential to increase the viability of lactic acid bacteria (*B. longum* and *L. acidophilus*) and decrease the viability of pathogenic bacteria *E. coli* and *S. Typhimurium*. These two effects make the neem gum solution a potential candidate for prebiotic drinks. The increased viability of both lactic acid bacteria can lead to healthy digestion due to the balance of microflora in them. In addition, diseases that attack the digestive tract such as diarrhea and salmonellosis can be prevented.

CONCLUSIONS

Based on the results of the research conducted, it was concluded that neem gum solution was able to increase the number of lactic acid bacteria (*Lactobacillus acidophilus* and *Bifidobacterium longum*) and reduce the number of pathogens (*Escherichia coli* and *Salmonella Typhimurium*) in vitro. This shows the neem gum solution potential to be used as a constituent in prebiotic drinks.

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