



## RESEARCH ARTICLE

# Neem Gum (*Azadirachta indica*) Solution Potential for Improving Viability of *Bifidobacterium Longum* and *Lactobacillus acidophilus* Bacteria

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## ABSTRACT

Neem gum is a liquid that comes out of the stems of neem plants (*Azadirachta indica*) that has hardened and become crystals. The content of neem gum has prebiotic potential to be used as a source of nutrients for lactic acid bacteria. *Bifidobacterium longum* and *Lactobacillus acidophilus* are lactic acid bacteria classified as beneficial bacteria in the gastrointestinal tract. These bacteria can selectively digest the content of soluble polysaccharides in neem gum. This study aims to determine the potential of neem gum solution (*Azadirachta indica*) to the viability value of *B. longum* and *L. acidophilus* bacteria. The viability of *B. longum* and *L. acidophilus* bacteria was calculated using the MTT assay method. The results showed that the viability value of both bacteria exposed to neem gum solution at concentrations of 5% (b/v), 10% (b/v), and 20% (b/v) increase compared to negative controls that were not given exposure to neem gum solution. Based on the study results, it is concluded that neem gum is effective in increasing the growth of bacteria *B. longum* and *L. acidophilus*.

**Keywords:** *Prebiotics; MTT assay; Soluble polysaccharides; Bifidobacterium; Lactobacillus*

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## HIGHLIGHTS

- ❖ Neem gum comes from the native Indian plant, the neem plant, and has prebiotics.
  - ❖ The prebiotics in the gum can be used as a source of nutrients for lactic acid bacteria.
  - ❖ Viability of *B. longum* and *L. acidophilus* increases as concentration of neem gum increases.
  - ❖ Neem gum is effective in increasing the growth of bacteria *B. longum* and *L. acidophilus*.
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## INTRODUCTION

Neem (*Azadirachta indica*) is a plant native to India. India is why neem plants have Latin names ending in "Indica" (Kumar & Navaratnam, 2013). Indian society calls neem a wonderful tree because it has been used as a traditional medicine for generations. In addition to its distribution in African regions such as Sudan and Nigeria, the plant is also found in regions in South Asia and Southeast Asia such as Thailand, Sri Lanka, Malaysia, Myanmar, and Indonesia are also found (Pankaj et al., 2011; Hashmat et al., 2012). In Indonesia, it spreads in Java, Madura, Bali, and Lombok (Prianto et al., 2019). This neem plant is commonly

found on the roadside because it can easily be bred in sexual and vegetative ways. In addition, the neem plant can also quickly adapt to dry soil conditions. However, this plant is often considered a weed and abandoned. In addition, the neem plant produces abundant gum that seeps out through the bark of its stems. However, local Indonesians have not widely explored this gum (Hutajulu & Siregar, 2015).

Neem gum is a liquid (exudate) that comes out of the neem plant, hardened, and becomes crystalline. The main component of the gum is soluble polysaccharides with branching several types of monosaccharides in it, such as L-arabinose, L-fucose, D-galactose, D-xylose, D-glucose, D-mannose, and their derivatives such as D-glucuronic acid (Nussinovitch, 2010; Moniem et al., 2018).

The content of polysaccharides in neem gum can be used as a functional foodstuff. Functional foodstuffs are food ingredients that provide energy and nutrition and improve health. In addition to being a trend today, the need for functional foodstuffs is increasing in line with the increasing prevalence of body health diseases with high medical costs (Kusumayanti et al., 2016; Abbas, 2020). One of the functional foodstuffs is prebiotics. Prebiotics are water-soluble foodstuffs that are not able to be decomposed and digested by the body but can be used as substrates by lactic acid bacteria in the gastrointestinal tract. This is the reason why prebiotics earned the nickname "colonic foods" (Hardisari & Amaliawati, 2016; Afni et al., 2017).



**Figure 1.** The form of gum liquid coming out of the bark of the neem tree (Annaamalai et al., 2015)



**Figure 2.** Dried crystalline neem gum (Nussinovitch, 2010)

Growth of lactic acid bacteria (LAB) such as *B. longum* and *L. acidophilus* need 'colonic foods'. LAB are beneficial bacteria included in normal intestinal microflora, especially the colon. The statement can be proven by the presence of *B. longum* and *L. acidophilus* bacteria in the gastrointestinal tract of healthy humans (Yuniastuti, 2014). *B. longum* and *L. acidophilus* bacteria are referred to as 'beneficial' bacteria because the dominance of their growth can suppress pathogenic bacteria to balance the intestinal microflora. Suppose there is a decrease in the growth of *B. longum* and *L. acidophilus* bacteria. In that case, it can increase the number of pathogenic bacteria, e.g., *E. coli* bacteria. This bacterium is a typical bacterium of the gastrointestinal tract. However, it can become pathogenic when the number of these bacteria increases which can often cause infections in the gastrointestinal tract, such as diarrhea.

Worldwide, there are more than 2 billion cases of diarrhea per year. About 900,000 cases of diarrhea require hospital treatment and there are about 2.5 million cases of death per year due to diarrhea (Amin, 2015). In Indonesia, this infection is included in the top 10 highest diseases and is the fourth leading cause of death, with a pain rate of 27% (Hatta, 2020; Ministry of Health, 2020).

The prevention of a high incident rate of diarrhea can be achieved by consuming functional foods such as prebiotics. Ingredients belonging to the prebiotic category usually contain polysaccharides and oligosaccharides which are complex carbohydrates (Lestari et al., 2018). The material is able to selectively stimulate the growth of *B. longum* and *L. acidophilus* because the bacteria have a particular enzyme, glycosyl hydrolase, which can hydrolyze complex polysaccharides into various constituent monosaccharides. The content will then be metabolized and used as energy to grow more (Husna et al., 2018). The growth of *B. longum* and *L. acidophilus* bacteria can suppress pathogenic bacteria in the gastrointestinal tract because they produce compounds such as SCFA (Short Chain Fatty Acid) that can interfere with the growth of pathogenic bacteria. Therefore, increased growth of bacteria *B. Longum* and *L. acidophilus* causes pathogenic bacteria to be depressed. It also balances the number of bacteria in the gastrointestinal tract to maintain its health (Hartono et al., 2012).

## MATERIAL AND METHODS

### Materials

The materials used in this study were crystal neem gum purchased from Baluran, Bayuwangi, East Java, *Bifidobacterium longum* FNCC 0210, *Lactobacillus acidophilus* FNCC 0051, medium deMain, Rogosa, Sharpe (MRS) agar Merck 1.10660.0500, MRS broth Merck 1.10660.0500, MTT (thiazolyl blue tetrazolium bromide) assay kit Biovision, commercial inulin (Orafti), anaeroGen, HCl, HCl cysteine, Aquades, aluminum foil, matches, sealer, and alcohol.

### Methods

This study aims to determine the potential of neem gum solution in improving the viability of *B. longum* and *L. acidophilus* bacteria in-vitro using the MTT assay method. The type of this research is an experimental laboratory with the *post-test of only the control group design* (Meilawaty, 2020).

#### *Making MRS Agar and MRS Broth*

MRS powder that had been weighed as much as 1.04 grams added L-cysteine HCl 0.01 grams then dissolved in 20 ml Aquades and heated using hotplates and magnetic stirrers. L-cysteine HCl was added to make the media environment more anaerobic. Each media pH was adjusted to six, adjusting the colon pH. The media was then sterilized in the autoclave at a temperature of 121° C for 15 minutes. The manufacture of MRS broth was made the same as the manufacture of MRS above. In this study, MRS broth without any added solution acts as a negative control. It is followed by the sterilization of MRS broth along with the neem gum that has been mixed into the media (Anindita, 2017; Widodo, 2019).

#### *Rejuvenation and suspension of B. longum and L. acidophilus bacteria*

Isolates of bacteria *B. longum* and *L. acidophilus* were re-bred on a Petri dish containing MRS agar. It was then incubated using an incubator for 24 hours at an anaerobic temperature of 37° C. After incubation, the bacteria were taken one colony and suspended on the media MRS broth that had been made and then incubated back the same as the previous state (Wahyuni, 2014).

*Making neem gum solution and control solution*

The crystals of neem gum that had been obtained were washed thoroughly and dried. Then, they are pounded until they became powder and sifted using a sieve to make it smooth. Neem gum powder was mixed with MRS broth at 60°C for easy solubility (Kamaraj et al, 2018). Commercial inulin which acts as a positive control was also mixed with MRS broth at the same temperature. The concentration of neem gum and positive control was made each 5% (b/v), 10% (b/v), and 20% (b/v) into the 1 ml of prepared MRS broth media. The solution was sterilized first using an autoclave at a temperature of 121°C for 15 minutes (Bajury, 2018).

*Bacterial inoculation and calculation of bacterial viability of B. longum and L. acidophilus using MTT (3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay*

The suspension of the two bacteria was each inoculated on a well plate of 50 µl and adapted in advance for 24 hours. Then, 50 µL of neem gum solution is inserted into a well plate containing bacteria. After that, it was incubated for 24 hours at an anaerobic temperature of 37°C (Aristyawan et al., 2016; Sun, 2016). Next, MTT reagents were added as much as 50 µL to the well plate and incubated for 3 hours for a reduction reaction. The administration of 150 µL of the MTT solvent to dissolve the formazan was done after a color change from yellow to purple. The microplate was then covered using aluminum foil and incubated in a shaker for 15 minutes. Then, the absorbance of live bacteria is read using an ELISA reader machine with an optical density of 595 nm (Hegyí et al., 2012; Benov, 2019). Absorbance obtained was then included in the viability formula as follows (Sun et al., 2016):

$$\% \text{ Cell viability} = \frac{\text{absorbance of treatment groups} - \text{media absorption}}{\text{control absorbance} - \text{media absorption}} 100\%$$

Where,

% Cell viability	: Percentage of living cells
Treatment absorbance	: Optical density (OD) value for each sample after treatment
Media absorption	: OD value formazan media control
Absorbance Cell control	: OD value formazan at average of cell controls

*Data analysis*

The research data were analyzed using the normality test and homogeneity test. The normality test using Shapiro Wilk showed that all samples had a value ( $p > 0.05$ ) so that the research data were normally distributed. The homogeneity test was continued using the Levene test with the results showing the data ( $p < 0.05$ ) is not homogeneous. So, the Kruskal-Wallis test was used and it showed the significant value is ( $p < 0.05$ ). Furthermore, the tests were carried out between the samples group using the Mann-Whitney test to determine the sample groups' significance value. The test results showed that almost all samples had a significant difference in value ( $p < 0.05$ ). Hayati (2020) mentioned that this showed a positive correlation and relationship between the neem gum solution and the growth of *B. longum* bacteria. Therefore, as the neem gum solution increases, the growth of *B. longum* bacteria will also increase.

**RESULTS**

**Results of bacterial viability calculation of MTT assay method**

Cell control shows the smallest absorbance value, indicating little growth of living bacteria. Its viability was 100% in both bacteria (Table 1). The higher the absorbance value, the higher the viability value obtained. The viability of *B. longum* bacteria in positive control exposed to inulin solution was 314.5% and 229.60% in *L. acidophilus* bacteria. The viability of *B. longum* bacteria in the neem gum treatment group was 219.5%, 328.7%, and 448.3% respectively and the viability of *L. acidophilus* bacteria was 189%, 232.8%, and 242.4% respectively.

**Table 1.** Average absorbance value and viability value

No	Research Group	Absorbance Value		Viability (%)	
		<i>B. longum</i>	<i>L. acidophilus</i>	<i>B. longum</i>	<i>L. acidophilus</i>
1	K -	0,2362	0,216	100	100
2	K +	0,546	0,378	314,5	229,6
3	P1	0,4088	0,328	219,5	189,
4	P2	0,5664	0,382	328,7	232,8
5	P3	0,7392	0,394	448,3	242,4

Where,

K - : Negative control/cell control (without treatment)

K + : Positive control (Inulin 10% (b/v))

P1 : Neem gum solution concentration 5% (b/v)

P2 : Neem gum solution concentration 10% (b/v)

P3 : Neem gum solution concentration 20% (b/v)

**DISCUSSION**

The results of the MTT assay test showed that the viability of bacteria in a solution of neem gum was more significant than the negative control. The negative control in this study was used as a growth control and the benchmark of the research group because these bacteria were not treated and only grown in their culture media. It had the lowest absorbance value with the viability value following the lowest. The positive control in this study was bacteria given a commercial inulin solution (Orafti). Inulin contains soluble polysaccharides with the constituents of fructose monosaccharides. Setiarto (2017) and Jora (2021) stated that the content of inulin foodstuffs cannot be digested by digestive system enzymes but can be used selectively as nutrients by *B. longum* and *L. acidophilus* bacteria to increase their amounts through their particular enzyme. The viability of the positive control in both bacteria had a greater value than the negative control. This indicated an increase in bacterial viability due to the presence of inulin solution.

The viability of both bacteria treated with neem gum solution had an upward tendency. This can be due to the increase in the content of neem gum polysaccharides in line with the increase in the concentration of each treatment. The research conducted by Herlina (2020) shows that the higher the concentration of water-soluble polysaccharides given, the more bacteria *B. Longum* and *L. acidophilus* were

obtained. For example, at neem gum concentration of 20%, there is more polysaccharides content than the concentration of 10% and 5%, so its ability to increase the viability of bacteria *B. Longum* and *L. acidophilus* is much greater. Increased viability indicates that *B. longum* and *L. acidophilus* bacteria are able to utilize heteropolysaccharide substrates in neem gum solutions. This research used the MTT assay method, which had a high level of thoroughness. The increase in viability of both bacteria was already seen in the concentration of neem gum solution of 5% in this method.

MTT assay tests showed that neem gum solution produced the viability of *B. longum* bacteria and large *L. acidophilus* bacteria. The greatest viability was obtained at a concentration of 20%, followed by a solution of neem gum concentration of 10%, then positive control containing inulin 10%, then a solution of neem gum concentration of 5%, and the smallest was the negative control. The growth of bacteria produced from the negative control group was the smallest because *B. Longum* bacteria and *L. acidophilus* bacteria did not get enough source of nutrients or 'food,' so they did not grow maximally.

The positive control used was inulin with a concentration of 10%. Isnasari (2020) suggested that inulin can be digested by *B. longum* bacteria using the enzyme  $\beta$ -fructofuranosidase. This enzyme will help *B. longum* bacteria to hydrolyze complex compounds from inulin to their constituent monosaccharides. Administration of neem gum solution 10% and 20% resulted in higher viability of *B. longum* bacteria than inulin. This ability is possible because neem gum has more varied types of constituent monosaccharides such as D-glucose, D-galactose, D-glucuronic acid, L - arabinose, L - fucose, D- mannose, and D- xylose (Moniem, 2018). In contrast, inulin mainly contains only one type of monosaccharide, namely fructose. Therefore, the content of neem gum can be maximally hydrolyzed by enzymes belonging to bacteria *B. longum* such as  $\alpha$ -glucosidase,  $\alpha$ -fucosidases,  $\alpha$ -arabinofuranosidase,  $\beta$ -arabinofuranosidase,  $\beta$ -galactosidase,  $\beta$ -mannosidase,  $\beta$ -xylosidase (Purwandani et al., 2018; Zabel,2020; Kelly, 2021). This causes both bacteria to get more nutrient intake in neem gum (Setiarto et al., 2017). However, the viability of *B. longum* and *L. acidophilus* bacteria in positive control was higher when compared to the 5% neem gum solution. This was possible because the positive control contained larger polysaccharide compounds that contained more nutrients when compared to neem gum solution of 5%.

The bacterial enzyme of *B. Longum* hydrolyzed the polysaccharides content in neem gum to become a simpler compound such as monosaccharides. This was done by breaking the glycosidic bond between parts using a particular enzyme belonging to the bacteria *B. Longum*. The monosaccharides would then be metabolized. Metabolism of neem gum by *B. Longum* and *L. acidophilus* bacteria produces SCFA (short-chain fatty acid) compounds. Mintarti (2017) suggests that the acid compound can diffuse into pathogenic bacterial cells and cause more acidic intracellular pH. This lower pH can result in the inactivation of enzymes in the intracellular and interfere with the transport of amino acids used by bacteria for protein synthesis. In addition, SCFA also interferes with the permeability of bacterial cell membranes, disintegrating the cell membranes. In addition to SCFA, the metabolism produces ATP used by these bacteria as energy to grow more (Kelly, 2021). The increased growth of *B. Longum* and *L. acidophilus* bacteria can prevent the adhesion of colonization of pathogenic bacteria by attaching to the enterocyte cell-binding site of the host's intestinal wall. Enterocyte cells that have been lectric by *B. Longum* and *L. acidophilus* bacteria cannot be lectric by pathogenic bacteria (Senditya et al., 2014). In addition, these bacteria maintain barrier function in the colon due to the production of antibacterial substances such as hydrogen peroxide and bacteriocin that will suppress pathogenic bacteria (Reivere, 2016). The decrease in the number of pathogenic bacteria will make *B. Longum* and *L. acidophilus* bacteria dominate the population in the gastrointestinal tract. This can awaken the health of the gastrointestinal tract from exposure to pathogenic bacteria (Hartono et al., 2012; Hardisari & Amaliawati, 2016).

Testing using the MTT assay method has several advantages, such as more sensitive calculations compared to the colony counting method, which can be wrong in its calculation. Since it is incorrect to calculate a collection of bacterial cells that are considered a single colony, the calculation results do not

show the actual number of bacteria (Soesetyaningsih & Azizah, 2020). Rosmania (2020) mentioned that this method takes a long time to prepare up to calculate the number of colonies. Another case with the MTT assay test is that it is an accurate test with high sensitivity because, in this method, the calculation of bacterial growth is seen based on the absorbance of living cells that are read objectively through an ELISA reader machine. The growth of live bacteria is seen by detecting the presence of metabolic activity in living cells. According to Mahfur (2016), dead bacteria cannot affect readings because they do not metabolize. In addition, MTT tests can quickly test samples simultaneously with minor sample requirements, making them more effective and efficient (Requena et al., 2019).

The explanation above shows that neem gum solution has the potential to increase the viability of *B. longum* and *L. acidophilus* bacteria with the presence of content in it. The increased viability of these two bacteria can cause healthy digestion due to the promotion of the balance of microflora in them.

## CONCLUSION

Results of the research showed that neem gum solution has the potential to increase the viability value of *B. longum* bacteria and *L. acidophilus* bacteria in vitro. It means that neem gum solution is a potential prebiotic drink; however, this needs further research to confirm this hypothesis. Further studies of neem gum solution can be conducted on other lactic acid bacteria or mice species.

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