

## Research article

Effect of a mannose to the interactions between *Naegleria fowleri* and pathogenic bacteria

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(Received: May 2021      Revised: June 2021      Accepted: July 2021)

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## ABSTRACT

**Introduction and Aim:** In this study, the interaction between pathogenic *Naegleria fowleri* and pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis* and *Salmonella typhi* was analyzed by a monosaccharide of mannose. Moreover, since the form of *N. fowleri* was found in diseases as cysts, the interaction between cysts and bacteria was analyzed.

**Materials and Methods:** In order to analyze the role of a monosaccharide called mannose in bacterial interaction, the analysis of bacterial association, invasion, and survival for amoeba treated with mannose was performed. *N. fowleri* trophozoites or cysts were pre-treated with a mannose at a concentration of 10, 50 and 100 mM for 1 hr at 37°C.

**Results:** The MRSA association was hardly suppressed until the concentration of mannose was 50 mM, but its association was reduced by about 168% to *N. fowleri* trophozoites by 100 mM mannose. Compared to the results for MRSA, the association of *E. faecalis* had little effect by mannose. Very interestingly, although *S. typhi* showed much higher invasion than the above MRSA and *E. faecalis*, it did not survive at all within *N. fowleri* trophozoites. Ten mM mannose showed a nearly similar 1% association with *N. fowleri* cyst treated, but not with 50 mM and 100 mM mannose treated *N. fowleri* cyst at all.

**Conclusion:** The association and invasion of *S. typhi* was highest for *N. fowleri* trophozoites and cysts, but the three bacteria did not survive in *N. fowleri* trophozoites and cysts.

**Keywords:** *Naegleria fowleri*; interaction; MRSA; *Enterococcus faecalis*; *Salmonella typhi*.

## INTRODUCTION

*Naegleria fowleri*, a brain-eating amoeba, is a protozoan that induces primary amoebic encephalitis in the cerebrum through the epithelial cells of the nose in humans and experimental animals and causes a very high mortality rate (1,2). *N. fowleri*, such as *Acanthamoeba*, can help bacteria such as *Legionella pneumophila* to grow in the cytoplasm. However, it has been reported that environmental microorganisms exist in the cytoplasm of the non-pathogenic *Naegleria* strain but reports on the exact life cycle or survival of the microorganism against the pathogenic amoeba, *N. fowleri*, are insufficient (3,4).

When *N. fowleri* destroys host cells, it binds to the host cells. *N. fowleri* destroys the host cell by the proteolytic enzyme secreted by *N. fowleri*, to proliferate and invade in central nervous system (CNS) (2,5) and can express amoeba structures such amoebastome that can be physically contacted to host cells (6,7). There are certain factors that trigger the adhesion of organisms, such as pore-forming proteins, the presence of carbohydrate residues on the outer surface of the plasma membrane, and the presence of terminal -L-Fucose and -D-glucose in glycol conjugates (2,8).

*Acanthamoeba*, a free-living amoeba similar to *Naegleria*, is known to interact with several bacteria such as *Escherichia coli* K1 (a bacterium that causes

meningitis) (9-11) and *L. pneumophila* (a bacterium that causes Legionella disease) (12). These bacteria can act as food for *Acanthamoeba* and contain some kind of polysaccharide layer (e.g., cell wall). The adhesion is a very important first step in amoeba's attacking host cells. It has been reported that non-pathogenic amoebae are less capable of binding to host cells (13). Contact-dependent interactions of these monosaccharide-binding proteins or monosaccharides itself may clarify the pathogenicity or infectivity of the free-living amoeba (9,11). In the previous report, the mannose-binding protein of *A. culbertsoni* could act as a very important factor in the interaction between amoeba and target cells (14).

The association between *A. culbertsoni* pretreated with polyclonal serum for a mannose-binding protein (MBP) and pathogenic bacteria such as *E. coli* O157:H7, *Staphylococcus aureus* and *Bacillus subtilis* was found to be reduced by about 35 to 40%, and monoclonal antibodies also showed similar results (15). To understand the interaction between bacteria and free-living amoeba in this way, it is necessary not only to study the contact, but also to analyze the invasion of bacteria and the survival of bacteria within the amoeba in more detail. Therefore, in this study, the interaction between pathogenic *N. fowleri* and pathogenic bacteria such as methicillin-resistant *S. aureus* (MRSA), *Enterococcus faecalis* and *Salmonella typhi* was

analyzed. In addition, since mannose sugar acted as a very important ligand in the *Acanthamoeba* study, it was tried to determine what changes occurred in the interaction with bacteria by treating mannose sugar in *N. fowleri*. Moreover, since the form of *N. fowleri* was found in diseases as cysts, the interaction between cysts and bacteria was analyzed.

## **MATERIALS AND METHODS**

### **Culture of *N. fowleri* trophozoites and inducement of *N. fowleri* cysts**

Unless otherwise stated, all reagents and laboratory materials were purchased from Sigma (Seoul, Korea). *N. fowleri* (Carter NF69 strain, ATCC No. 30215) trophozoites were aseptically cultured at 37°C in Nelson's medium (1.7 g neutralized liver digest (Oxoid, Basingstoke, England), 1.7 g glucose, 4 mg MgSO<sub>4</sub>·7H<sub>2</sub>O, 4 mg CaCl<sub>2</sub>·2H<sub>2</sub>O, 142 mg Na<sub>2</sub>HPO<sub>4</sub>, 136 mg KH<sub>2</sub>PO<sub>4</sub>, 120 mg NaCl, 10% fetal bovine serum (ThermoFisher Scientific, Seoul, Korea) (16). The medium was exchanged when the trophozoites became 70% confluent by periodically observing it under an inverted microscope. For the analysis of bacterial interactions against *N. fowleri* cysts, cysts were induced from *N. fowleri* trophozoites. This method was previously performed by referring to the method used for the cyst induction of *Acanthamoeba* (11). *N. fowleri* trophozoites were transferred to a 3% non-nutrient agar plate (Oxoid limited, Seoul, Korea), and the plate was incubated for up to 4 days at 30°C. Cysts forming groups were periodically observed under a bright microscope. After addition of phosphate-buffered saline (PBS), the surface of the agar was gently scraped off using a cell scraper to finally confirm the formation of cysts.

### **Culture of bacteria**

Three pathogenic bacteria such as MRSA (ATCC No. BAA-1769), *E. faecalis* (KCTC No. 5290) and *S. typhi* (ATCC No. 19430) were applied in the interaction with *N. fowleri* trophozoites and cysts.

The number of bacterial colonies was calculated for the interaction analysis. Therefore, all bacteria were cultured using tryptic soy agar (TSA, MB cell, Korea), which was a generally used enrichment medium instead of the bacterial selection medium. Bacteria were cultured at 37°C, and the bacteria were once again confirmed through Gram staining (17). Since bacterial colonies were accurately calculated and added to the amoeba above, the following experiment was performed by adjusting the McFarland turbidity of 0.5 representing 0.5 x 10<sup>3</sup> to 1.5 x 10<sup>6</sup> colony forming units (cfu)/mL.

### **Bacterial association, invasion assay and survival assay**

It is known that *N. fowleri* is capable of preying and binding bacteria but reports of how bacteria interact with *N. fowleri* are still weak. In previous studies on *A. culbertsoni*, a free-living amoeba, the mannose binding

protein (MBP) played a very important role in interactions with host cells. Therefore, in this study, to analyze the role of a monosaccharide called mannose in bacterial interaction, the analysis of bacterial association, invasion, and survival for amoeba treated with mannose was performed. All experimental methods have been changed slightly from a previous report (11). *N. fowleri* trophozoites or cysts were pre-treated with a mannose at a concentration of 10, 50 and 100 mM for 1 hr at 37°C and the washed two times with PBS. All bacteria were added to *N. fowleri* trophozoites or cysts by adjusting to a 0.5 McFarland index. Briefly, *N. fowleri* trophozoites or cysts were incubated in a 24-well culture plate with Nelson medium. *N. fowleri* trophozoites or cysts pre-treated with mannose was incubated with bacteria (2 x 10<sup>5</sup> cfu / 0.5 ml of PBS) at room temperature (RT) for 1 hr. After washing three times with PBS, SDS (final concentration 0.5%) was added to each well for 30 min to destroy *N. fowleri*, and the number of bacterial colonies produced was calculated by incubating in a TSA plate for one day. The result obtained is called bacterial association and the calculation method is as follows: recovered bacteria (cfu) / total bacteria (cfu) × 100 = % of bacteria associated with *N. fowleri*. Since the bacteria would associate with *N. fowleri* cell wall and then invade, the invasion assay proceeded as follows. After the association analysis, gentamicin antibiotics was added for 45 minutes (100 µg/ml, final concentration) to remove bacteria not associated with *N. fowleri*. The result obtained was expressed as the number of colonies of the recovered bacteria relative to the total colonies of the invading bacteria as shown in the above association result. The plate after the invasion assay was placed at 37°C for 24 hr to see how much of the invading bacteria survived within *N. fowleri*. Thereafter, the supernatant was removed, and colonies of bacteria cultured in TSA for one day were calculated to calculate the survival of bacteria. The calculation method evaluated the number of bacteria recovered as described above.

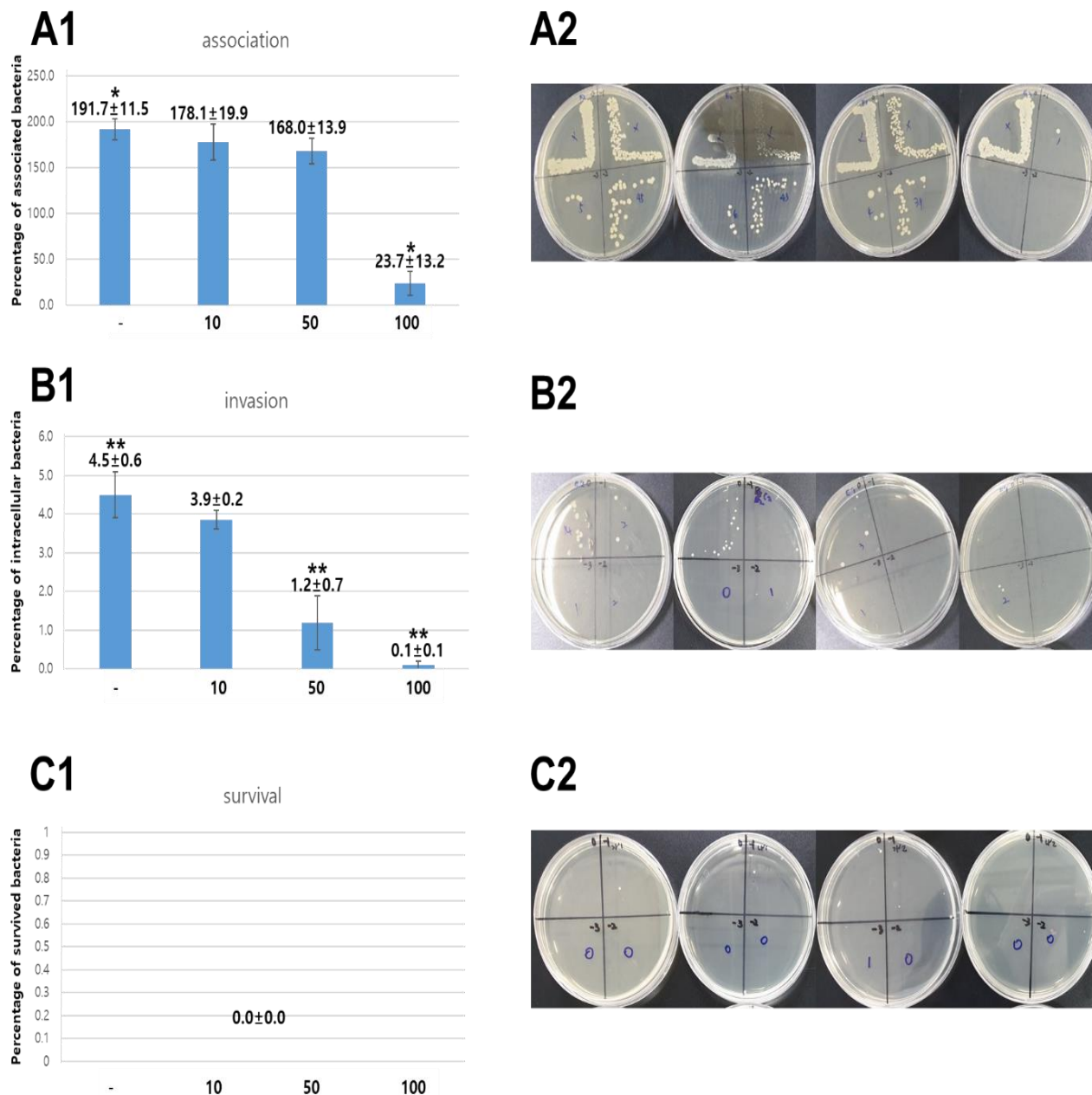
### **Statistical analysis**

To determine the statistical significance of the resulting values of the interaction, Student two-sample t test was performed. If P < 0.05, it was determined to show statistical significance.

## **RESULTS**

### **Effect of mannose on *N. fowleri* trophozoites in the interaction with MRAS**

In the study of *Acanthamoeba*, MBP was known to play an important role in the contact-dependent pathway. Therefore, in this experiment, it was analyzed how mannose binding to MBP affected the interaction between *N. fowleri* trophozoites and MRSA, namely, bacterial association, invasion, and survival. As shown in the results of Fig. 1, it was observed that *N. fowleri* trophozoites, treated with a high concentration of 100 mM mannose, inhibited the interaction with bacteria.



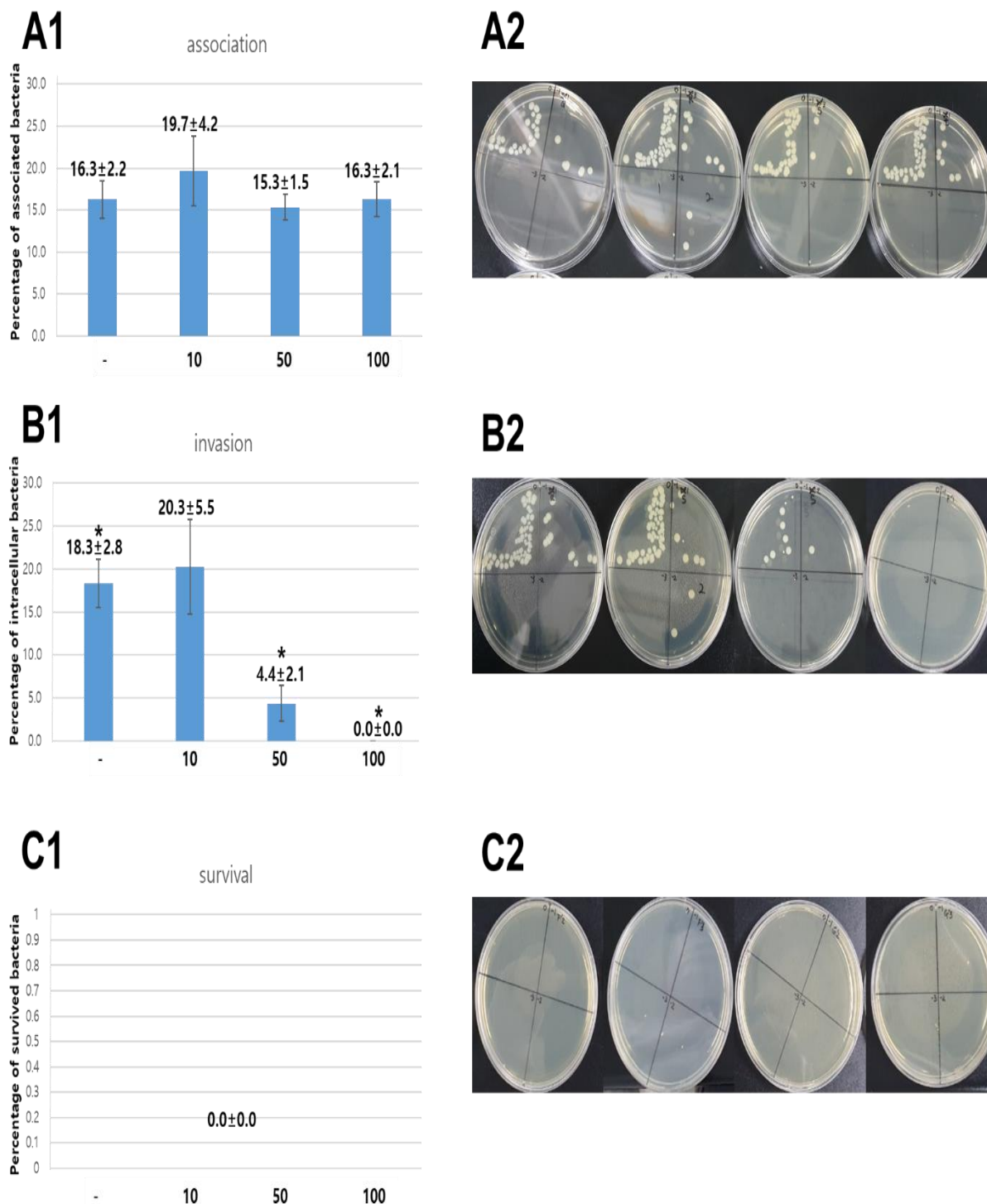
**Fig. 1:** MRSA association, invasion, and survival to *N. fowleri* trophozoites. A1, B1 and C1 represented the percentage of MRSA association, invasion, and survival of the bacteria, respectively, and A2, B2 and C2 represented the plates for A1, B1 and C1, respectively. The number on the X-axis indicated the mM concentration of mannose in A1, B1 and C1. This experiment was performed in triplicate wells with three times, and data were indicated by standard deviation (SD) value.

In a more detailed analysis, the results of bacterial association showed that the bacterial association was hardly suppressed until the concentration of mannose was 50 mM, but the association of bacteria was reduced by about 168% with respect to untreated *N. fowleri* trophozoites by 100 mM mannose (Fig. 1 A1, B1) ( $P < 0.05$ ). On the other hand, there was not much difference between the results of mannose on the invasion results compared to the results of bacterial association (Fig. 1 A2, B2). The invasion of MRSA was reduced by about 4.3% and 4.4% for untreated *N. fowleri* trophozoites by 50 mM and 100 mM mannose, respectively. However, assuming that the invasion of MRSA without mannose treatment was 100%, its invasion was decreased by about 73.4% by 50 mM

mannose and about 97.8% by 100 mM mannose ( $P < 0.05$ ). Interestingly, MRSA did not survive in *N. fowleri* trophozoites at all compared to *N. fowleri* trophozoites treated with mannose from the control group (Fig. 1 A3, B3).

**Effect of mannose on *N. fowleri* trophozoites in the interaction with *E. faecalis***

*E. faecalis* belonging to Group D *Streptococcus*, is a bacterium that lives in the human gastrointestinal tract. In order to analyze the interaction between *E. faecalis* and mannose treated *N. fowleri* trophozoites, it was carried out by the same method as MRSA mentioned above (Fig. 2).

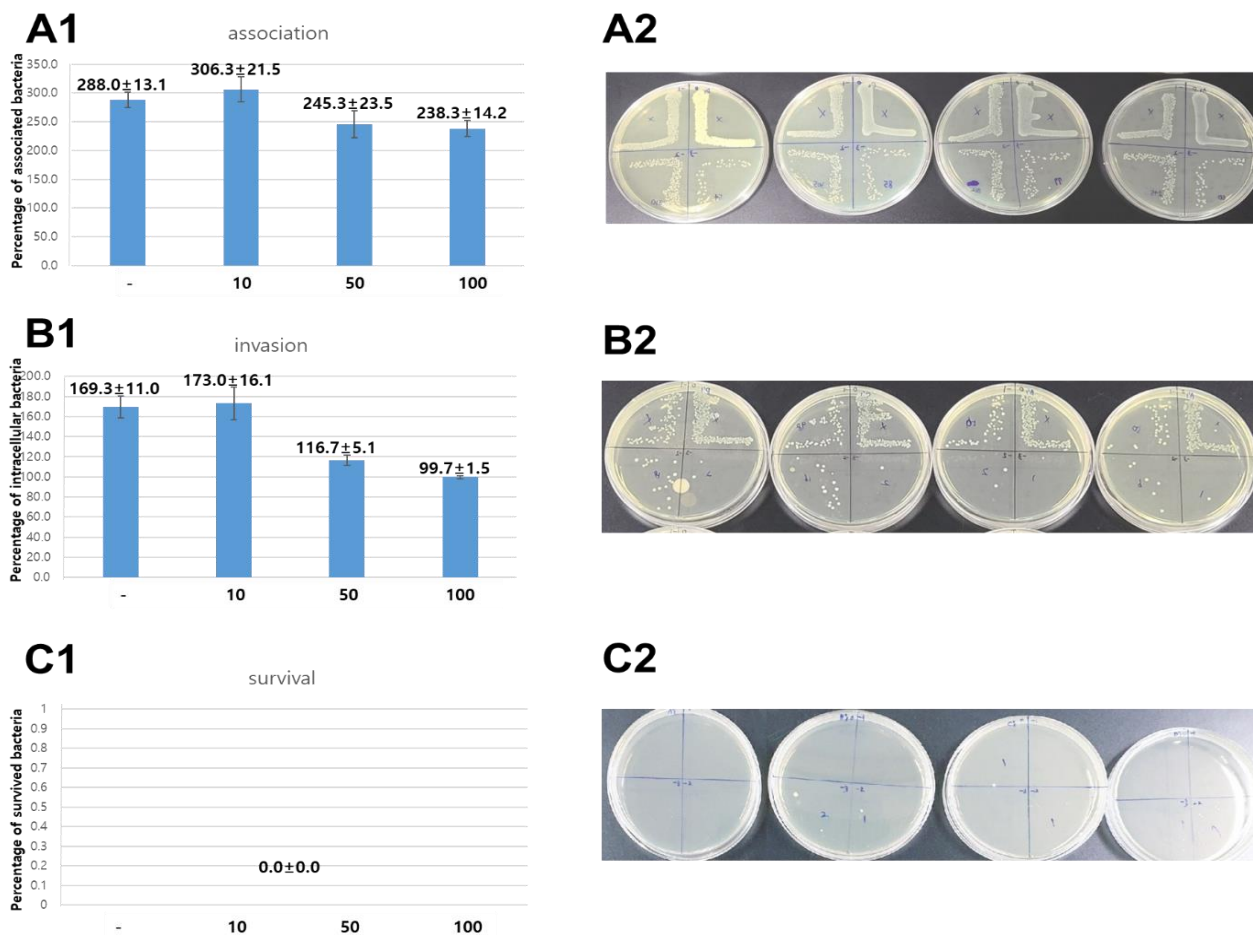


**Fig. 2:** *E. faecalis* association, invasion, and survival to *N. fowleri* trophozoites. A1, B1 and C1 represented the percentage of *E. faecalis* association, invasion, and survival of the bacteria, respectively, and A2, B2 and C2 represented the plates for A1, B1 and C1, respectively. The number on the X-axis indicated the mM concentration of mannose in A1, B1 and C1. This experiment was performed in triplicate wells with three times, and data were indicated by SD value.

Compared to the results for MRSA, the association of *E. faecalis* had little effect by mannose (Fig. 2 A1, B1). However, the invasion of *E. faecalis* was reduced by about 14% with 50 mM mannose than when mannose was not treated (Fig. 2 A2, B2) ( $P < 0.05$ ). In addition, invasion of *E. faecalis* to *N. fowleri* trophozoites pre-treated with 100 mM mannose did not occur at all ( $P < 0.05$ ). *E. faecalis* did not survive in *N. fowleri* trophozoites at all, very similar to the results of MRSA (Fig. 2 A3, B3).

**Effect of mannose on *N. fowleri* trophozoites in the interaction with *S. typhi***

There was no statistically significant difference compared to the association between MRSA and *E. faecalis* and the results of invasion, but the association and invasion of *S. typhi* were much higher than those of the other two bacteria such as MRSA and *E. faecalis* (Fig. 3).



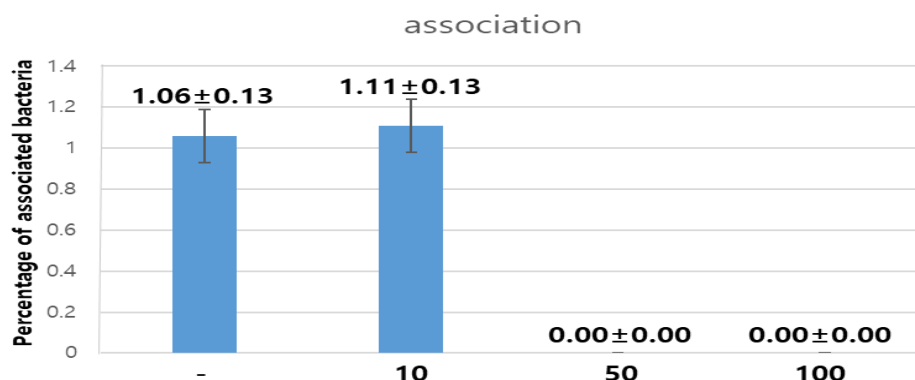
**Fig. 3:** *S. typhi* association, invasion, and survival to *N. fowleri* trophozoites. A1, B1 and C1 represented the percentage of *S. typhi* association, invasion, and survival of the bacteria, respectively, and A2, B2 and C2 represented the plates for A1, B1 and C1, respectively. The number on the X-axis indicated the mM concentration of mannose in A1, B1 and C1. This experiment was performed in triplicate wells with three times, and data were indicated by SD value.

*S. typhi* was almost 238% to 306% associated with *N. fowleri* trophozoites regardless of mannose-treated *N. fowleri* trophozoites (Fig. 3 A1, B1). On the other hand, *S. typhi* invasion was reduced by 52.6% in 50 mM mannose and 69.6% in 100 mM mannose compared to the control when mannose was treated. Very interestingly, although *S. typhi* showed much higher invasion than the above MRSA and *E. faecalis*, it did not survive at all within *N. fowleri* trophozoites.

**Effect of mannose on *N. fowleri* cysts in the interaction with *S. typhi***

*N. fowleri* cysts have less motility than *N. fowleri*

trophozoites, but the thickness of its cell wall is thicker than trophozoite type. However, rather than the *N. fowleri* cyst, *Acanthamoeba* cyst has a very thick double membrane. In this experiment, the interaction between *S. typhi* and *N. fowleri* cyst, which showed the highest association and invasion, was analyzed. As shown in Fig. 4, compared to the control group, 10 mM mannose showed a nearly similar 1% association with *N. fowleri* cyst treated, but not with 50 mM and 100 mM mannose treated *N. fowleri* cyst at all. On the other hand, *S. typhi* invasion and survival were all 0%, so it was not shown in this figure.



**Fig. 4:** *S. typhi* association to *N. fowleri* cysts. This experiment was performed in triplicate wells with three times, and data were indicated by SD value. The number on the X-axis indicated the mM concentration of mannose.

## DISCUSSION

Among the free-living amoebae, *Acanthamoeba*, which has been well analyzed for interaction with bacteria such as *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, etc., could interact with various bacteria and bind to the bacterial cell wall to ingest bacteria. There is little analysis of the monosaccharide or polysaccharide effects of free-living amoebae, but one study found that highly interacting *L. pneumophila* was the  $\alpha$ 1-3D-mannobiose domain of the mannose-of *A. castellanii* (18). Moreover, *L. pneumophila* has high affinity with GalNAc $\beta$ 1-4Gal domain of the N-acetyl-D-galactosamine receptor of *N. lovaniensis*. Thus, monosaccharides or polysaccharides located outside the cell wall of bacteria would be able to bind to the glycosylated protein of *Naegleria* spp. In *N. fowleri*, there have been recent reports on the association of mannose and fucose with host cell adhesion and cytotoxicity (19). Therefore, the possibility of vaccine and diagnosis was suggested using lectin, a sugar-binding protein. In this study, it was analyzed with the association of some clinically important bacteria, e.g., MRSA, *E. faecalis*, *S. typhi*, with *N. fowleri* trophozoites treated with mannose, and analyzed the number of bacteria that could invade and survive after the bacteria bound with *N. fowleri* trophozoites. Furthermore, the interaction between mannose-treated *N. fowleri* cysts morphologically different from *N. fowleri* trophozoites and bacteria was also analyzed. Of the three bacteria analyzed above, the percentage of *S. typhi* interacting with *N. fowleri* trophozoites was the highest, but none of the bacteria survived within the cytoplasm of *N. fowleri* trophozoites and cysts. There was currently no report on the reason for supplementing these results, but it was thought that the bacteria entering and invading by various proteolytic enzymes in the cytoplasm of *N. fowleri* might be killed by incubating for 24 hr for survival evaluation. There was a report that explained the survival of bacteria within *Acanthamoeba* and the process by which *Acanthamoeba* consumed bacteria. The ability of *A. castellanii* to promote the survival of *Vibrio parahaemolyticus* POR1 strain could be promoted by factors secreted from *A. castellanii*, not by direct contact between *A. castellanii* and *V. parahaemolyticus* POR1 (20). This can be explained as chemotaxis. When *N. fowleri* trophozoites bound to target cells, it stimulated the mobility and proliferation of *N. fowleri* trophozoites due to the selective chemotactic factor. If it was reinforced by the downstream signals, the chemotaxis could be more and more reinforced (21-22). With binding assays using different lectins, *Naegleria* spp. showed the abundance of surface glycoconjugates containing  $\alpha$ -D-glucosyl,  $\alpha$ -D-mannosyl and  $\alpha$ -L-fucosyl, N-acetyl- $\alpha$ -D-galactosaminyl and  $\alpha$ -D-galactose residues (8, 23). On the other hand, the expression of monosaccharide residues in these glycoproteins had the potential to be evaluated as an index capable of distinguishing and diagnosing pathogenic and non-pathogenic *Naegleria*

(8). When biotinylated lectin was used, alpha-D-mannose, alpha-D-glucose, and terminal alpha-L-fucose residues were observed to be higher in pathogenic *N. fowleri* than in non-pathogenic *N. gruberi* (8). In another report, analysis of the difference in carbohydrate content between *N. fowleri* and *N. lovaniensis* showed thicker aggregates for mannose and galactose in *N. fowleri* than *N. lovaniensis* (24).

## CONCLUSION

Analysis of bacterial interactions on *Naegleria* is still inadequate. In this study, the association, invasion, and survival of mannose-treated *N. fowleri* trophozoites and cysts and bacteria such as MRSA, *E. faecalis* and *S. typhi* were analyzed using mannose, which was considered important for contact with target cells or bacteria in free-living amoebae. The association and invasion of *S. typhi* was highest for *N. fowleri* trophozoites and cysts, but the three bacteria did not survive in *N. fowleri* trophozoites and cysts. Therefore, this study suggests that mannose played an important role in association and invasion of *N. fowleri* and bacterial interactions. Therefore, this study may be helpful in analyzing how *N. fowleri* survives in the environment and its interaction with host cells.

## ACKNOWLEDGMENT

Funding for this paper was provided by Namseoul University.

## CONFLICT OF INTEREST

Author has no conflict of interest.

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