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Brief Communication

Interaction of *Naegleria fowleri* Trophozoites with *Escherichia coli* and MRSA by N-acetylglucosamine and Galactose

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Naegleria fowleri is a free-living amoeba causing primary amoebic encephalitis. In this study, we analyzed how the N-aceytlglucosamine (GlcNAc) and D-galactose affected the interaction between *Naegleria fowleri* and methicillin-resistant *Staphylococcus aureus* (MRSA) or *Escherichia coli* O157:H7, and the interaction with bacteria when monosaccharides were treated with *N. fowleri* for a longer pre-incubation time. When GlcNAc was treated with *N. fowleri* for 1 hr, the *E. coli* association was almost the same as that of the control not treated with GlcNAc until the concentration of GlcNAc was 25 mM. However, the *E. coli* association was reduced by approximately 91% with 100 mM GlcNAc. *E. coli* invasion into *N. fowleri* showed statistical significance only in the group treated with 100 mM GlcNAc. The interaction when treated with galactose showed a very different pattern in the 50 mM galactose group than when treated with GlcNAc. In the MRSA interaction, a statistically significant decrease in association (76.3% by GlcNAc and 88.7% by galactose) and invasion (3.6% by GlcNAc and 9.3% by galactose) was shown by the concentration of two 100 mM monosaccharides. The group treated with monosaccharides at the same time showed almost no difference in all interactions from the group treated with monosaccharides at the same time. Taken together, it suggested that the effect of monosaccharides on the interaction of several Gram-negative or positive bacteria and the evidence that the interaction could be enhanced by longer pre-incubation time.

Key Words: *Naegleria fowleri*, N-aceytlglucosamine, D-galactose, Bacteria

Naegleria fowleri is a free-living amoeba that lives in rivers and lakes, and is a brain-eating amoeba known to cause primary amoebic encephalitis (PAME) in humans and experimental animals (Siddiqui et al., 2016; Jahangeer et al., 2020; Jung, 2021). The form of *N. fowleri* changes according to environmental requirements or infection if there are trophozoite, cyst and flagellate (Guzmán-Téllez et al., 2020). Among them, the trophozoite is active in eating bacteria or food because of the active pseudopodial movement (Marciano-Cabral and Cline, 1987). Although there were reports that *N. fowleri* could feed on microorganisms and survive, reports on the detailed mechanism are still insufficient (Walochnik et al., 2005; Visvesvara et al., 2007). However, in the presence of L-fucose, D-glucose, and D-mannose, it can bind to carbohydrate residues on the plasma membrane surface and allow bacteria to attach (Cervantes-Sandoval., 2010; Jahangeer et al., 2020; Jung, 2021). Mannose is also well known in *Acanthamoeba* spp. as a free-

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living amoeba (Huth et al., 2017), and a recent study reported that mannose inhibited Methicillin-resistant *Staphylococcus aureus* (MRSA) association by nearly 168% (Jung, 2021).

In this study, we analyzed how the N-aceytlglucosamine (GlcNAc) and D-galactose (mentioned by galactose below) monosaccharides affected the interaction between *N. fowleri* trophozoites (mentioned by *N. fowleri* below) and MRSA or *Escherichia coli* O157:H7 (mentioned by *E. coli* below), and the interaction with bacteria when monosaccharides were treated with *N. fowleri* for a longer pre-incubation time.

N. fowleri (Carter NF69 strain, ATCC No. 30215) trophozoites were cultured aseptically at 37℃ using Nelson's medium (Willaert, 1971). It was continuously subcultured so that about 95% of the trophozoites, in which a lot of pseudopods were observed, was maintained. In this study, two pathogenic bacteria were used. One was gram-negative *E. coli* (ATCC No. 43895) and the other was gram-positive MRSA (ATCC No. BAA-1769). A selective medium for bacteria was not used, but tryptic soy agar (TSA, MB cell, Korea) was used to propagate the bacteria at 37℃. In addition, bacteria were confirmed once again through Gram staining (Lim et al., 1988). In order to accurately count the number of the bacteria, the bacteria were diluted with tryptic soy broth (TSB) and 0.85% NaCl in advance so that the McFaland turbidity of 0.5 showed 0.5×10^3 to 1.5×10^6 colony forming units (cfu)/mL (Song and Jung, 2017). To investigate the effect of GlcNAc (Merck, Suwon, Korea) and galactose (Merck, Suwon, Korea) on the interaction between *N. fowleri* and bacteria, the concentrations of each monosaccharide were treated with 10, 50, and 100 mM.

Analysis of the interaction between *N. fowleri* and *E. coli* or MRSA calculated how much the bacteria associated with *N. fowleri*, how much invasion into the *N. fowleri* and how long it survived within the *N. fowleri*. The experimental procedure was referenced by Jung et al. (2007) and several important procedures, e.g., pre-incubation time, the treatment of the detergent were changed. First, 10, 50 and 100 mM monosaccharides were treated to *N. fowleri* (5×10^4/well) with phosphate-buffered saline (PBS) for 1 hr in a 24-well cell culture plate at 37℃. This reaction time was a pre-incubation time, which was doubled compared to the 30 minutes used in previous papers. Each bacterium (2×10^5 cfu/0.5 mL of PBS) were reacted with *N. fowleri* cultured in wells at room temperature (RT) for 1 hr. After washing wells 3 times with PBS, SDS (0.5% final concentration) was added for 1 hr to completely dissolve *N. fowleri* instead of bacteria, and the number of bacteria was counted by plating on TSA. In this procedure, bacterial association percentages were calculated as follows: recovered bacteria (cfu)/total bacteria (cfu) × 100 = % bacteria associated with *N. fowleri*. Then, bacteria associated to *N. fowleri* can invade the *N. fowleri*. Therefore, it was analyzed how many bacteria invaded into *N. fowleri*. At first, gentamicin antibiotics (100 μg /mL, final concentration) was added for 45 min to remove the bacteria attached to or outside the *N. fowleri* cell wall. After washing wells 3 times with PBS, bacterial plating was done on TSA. The degree of bacterial invasion into the *N. fowleri* was calculated as follows: recovered bacteria (cfu)/ total bacteria (cfu) × 100 = percentage of invaded bacteria. Bacteria invading into *N. fowleri* can either survive in the amoebae or are killed by enzymes in other organelles such as vacuoles. By adding PBS as the growth medium for *N. fowleri*, how much bacteria survived within *N. fowleri* for 24 hr at 37℃ was also analyzed. After the reaction time was over, all of the supernatant was removed, washed three times with PBS, and then the bacteria were plated on TSA. The degree of bacterial survival within the *N. fowleri* was calculated as follows: recovered bacteria (cfu)/total bacteria (cfu) × 100 = percentage of survived bacteria. In all of the above processes, the monosaccharides were pre-reacted with *N. fowleri*, and then interactions with bacteria were analyzed. In addition, the interaction between *N. fowleri* and bacteria was analyzed by treating the monosaccharides while growing in the wells. For these reactions, association, invasion and survival were calculated using the formulas mentioned above.

Statistical significance was analyzed by comparing the control group untreated with monosaccharides and the experimental group treated with monosaccharides. Student two-sample *t*-test was performed using the mean and standard deviation (SD). The statistically significant difference was $P<0.05$.

The most important point in this study was to analyze how GlcNAc and galactose other than mannose (Huth et al., 2017), which were key in previous studies, affect the

interaction between *N. fowleri* and bacteria. Therefore, in this study, GlcNAc and galactose were pretreated with *N. fowleri* to saturate the *N. fowleri* cell wall with the above polysaccharide, and then, the interaction with bacteria was analyzed. Since it was confirmed that non-pathogenic bacteria had low interactions with free-living amoebae according to previous studies (Alsam et al., 2006), the interaction was analyzed using pathogenic bacteria such as *E. coli* or MRSA in this study. In addition, the effect of monosaccharide treatment on their interaction was analyzed when *N. fowleri* and bacteria were co-cultured. The interaction was to check whether bacteria were attached to *N. fowleri* and then invaded into *N. fowleri* and survived.

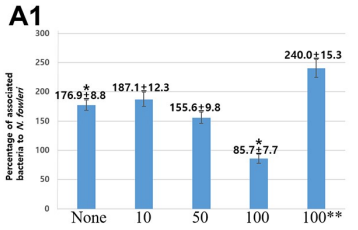
When GlcNAc was treated with *N. fowleri* for 1 hr, the *E. coli* association was almost the same as that of the control not treated with GlcNAc until the concentration of GlcNAc was 25 mM. Although not statistically significant, the *E. coli* association was reduced by about 11% when treated with 50 mM GlcNAc. However, the *E. coli* association was reduced by approximately 91% with 100 mM GlcNAc (Fig. 1, A1 and A2). In addition, when compared with the control group, there was a statistically significant difference from the group of the *E. coli* association treated with 100 mM GlcNAc. *E. coli* invasion into *N. fowleri* showed statistical significance only in the group treated with 100 mM GlcNAc, and the invasion was reduced by about 4.1% compared to the control group (Fig. 1, B1 and B2). One colony of *E. coli* survival within *N. fowleri* was found in the control group and the group treated with 10 mM GlcNAc, but the survival rate was close to zero (Fig. 1, C1 and C2). The interaction when treated with galactose showed a very different pattern in the 50 mM galactose group than when treated with GlcNAc (Fig. 1, D1, D2, E1, E2). In particular, in the group treated with 50 mM galactose, about 169.7% of *E. coli* association and about 9% of *E. coli* invasion were reduced, respectively, and showed statistical significance compared to the control group. The interaction was gradually decreased with galactose than with GlcNAc according to the concentration of galactose. *E. coli* survival percentagewas close to zero even with galactose (Fig. 1, F1, F2).

MRSA, a Gram-positive bacterium, was analyzed for interaction like *E. coli*. (Fig. 2). Overall, when analyzing Fig. 2, it was not confirmed that GlcNAc and galactose were gradually decreased according to the concentration. However, a statistically significant decrease in association (76.3% by GlcNAc and 88.7% by galactose) and invasion (3.6% by GlcNAc and 9.3% by galactose) was shown by the concentration of two 100 mM monosaccharides (Fig. 2, A1, A2, B1, B2, E1, E2). The survival rate within *N. fowleri* of MRSA was 0% without the effect of monosaccharides (Fig. 2, C1, C2, F1, F2).

All the above results were analyzed after pretreatment of monosaccharides with *N. fowleri* for 1 hr. The effect of monosaccharides was also analyzed if *N. fowleri* and bacteria were co-cultured described at groups marks with asterisks in Figure 1 and 2. The group treated with monosaccharides at the same time showed almost no difference in all interactions from the group treated with monosaccharides at the same time.

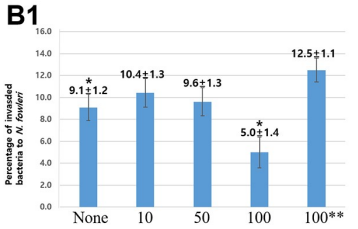
The *N. fowleri* trophozoite is a form that can eat bacteria or food well due to active psuedopodial movement (Marciano-Cabral and Cline, 1987). According to previous studies, it is known that pathogenic bacteria interact with *Acanthamoeba* much more than non-pathogenic bacteria (Jung, 2011). Such an interaction can be established by a contact-dependent mechanism, and monosaccharides can serve as an intermediary for such interactions (Khan, 2003). In addition, there are reports that mannose plays a very important role in the interaction between *Acanthamoeba* spp. and bacteria, but there are still insufficient reports on which monosaccharides play a specific role in *N. fowleri* belonging to the same free-living amoeba. However, it has not yet been reported how much the interaction differs depending on whether the bacteria are gram-negative or positive. In the experimental procedure of this study, GlcNAc or galactose was added to *N. fowleri* for 1 hr, whereas in other studies, 30 minutes was mainly used. This was intended to enhance the interaction a little more.

The invasion percentages of bacteria entering the *N. fowleri* were confirmed by treatment with gentamicin antibiotics. In this study, it could be mistaken for percentages that were much smaller than the association experimental groups. For example, 9.1% invasion in the control group was much smaller percentages than 176% association, but if



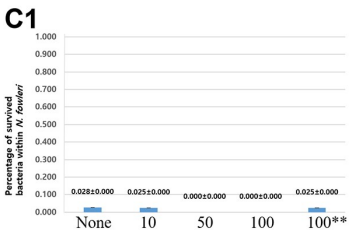
A2

Treatment of GlcNAc	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	130000	155000	180000	105000	125000
Bacterial colonies diluted 100-fold	92	116	112	36	120
Total bacterial colonies grown	230000	290000	280000	90000	300000



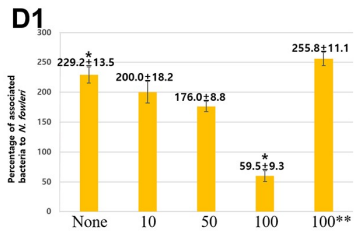
B2

Treatment of GlcNAc	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	110000	120000	130000	100000	100000
Bacterial colonies diluted 10-fold	40	50	50	20	50
Total bacterial colonies grown	10000	12500	12500	5000	12500



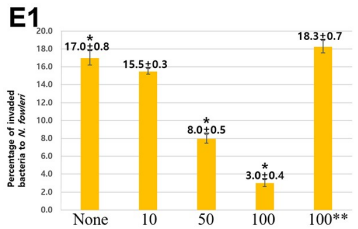
C2

Treatment of GlcNAc	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	90000	100000	100000	95000	100000
Bacterial colonies diluted 0-fold	1	1	0	0	1
Total bacterial colonies grown	25	25	0	0	25



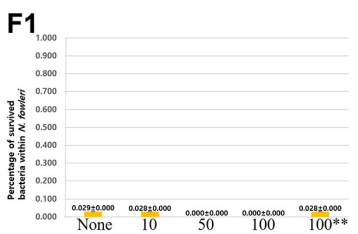
D2

Treatment of galactose	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	120000	160000	125000	105000	130000
Bacterial colonies diluted 100-fold	110	128	88	25	133
Total bacterial colonies grown	275000	320000	220000	62500	332500



E2

Treatment of galactose	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	100000	100000	110000	100000	100000
Bacterial colonies diluted 10-fold	68	62	35	12	73
Total bacterial colonies grown	17000	15500	8750	3000	18250

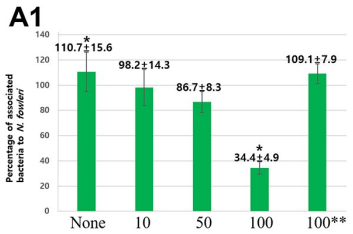


F2

Treatment of galactose	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	85000	89000	90000	90000	89000
Bacterial colonies diluted 0-fold	1	1	0	0	1
Total bacterial colonies grown	25	25	0	0	25

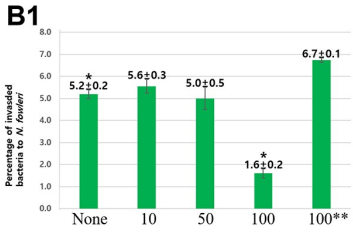
Fig. 1. Interactions between *N. fowleri* and *E. coli* by GlcNAc and galactose. A1 and D1 showed association, B1 and E1 showed invasion, and C1 and F1 showed survival results. A2, B2, C2, D2, E2, and F2 represented CFU obtained for calculating the values of the graphs in the left panels. The number below the bar graph indicated the concentration of monosaccharides. An asterisk (*) represented $P<0.005$. Other asterisks (**) showed the result of treating monosaccharides in co-cultured state of *N. fowleri* and *E. coli*.

only the invasion results were compared, the difference was more than double, so it could be judged that the difference between the invasion groups by GlcNAc or galactose was quite high. The parts marked with an asterisk in the experi-



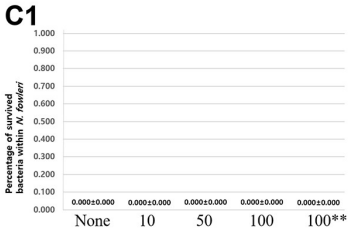
A2

Treatment of GlcNAc	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	140000	140000	150000	160000	165000
Bacterial colonies diluted 100-fold	62	55	52	22	72
Total bacterial colonies grown	155000	137500	130000	55000	180000



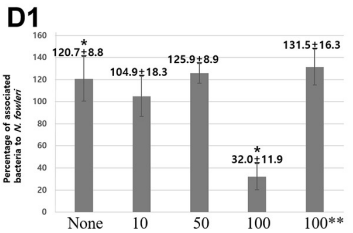
B2

Treatment of GlcNAc	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	135000	135000	130000	125000	115000
Bacterial colonies diluted 10-fold	28	30	26	8	31
Total bacterial colonies grown	7000	7500	6500	2000	7750



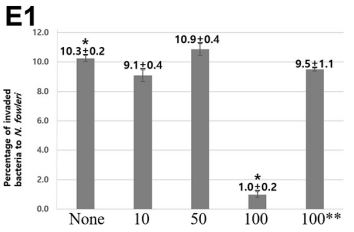
C2

Treatment of GlcNAc	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	80000	65000	87500	95000	84000
Bacterial colonies diluted 0-fold	0	0	0	0	0
Total bacterial colonies grown	0	0	0	0	0



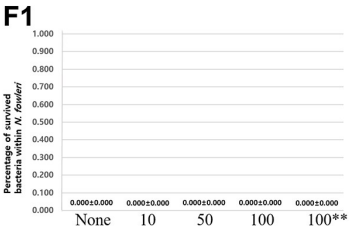
D2

Treatment of galactose	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	145000	162000	145000	125000	135000
Bacterial colonies diluted 100-fold	70	68	73	16	71
Total bacterial colonies grown	175000	170000	182500	40000	177500



E2

Treatment of galactose	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	100000	110000	99000	100000	100000
Bacterial colonies diluted 10-fold	41	40	43	4	38
Total bacterial colonies grown	10250	10000	10750	1000	9500



F2

Treatment of galactose	none	10 mM	50 mM	100 mM	100 mM**
Number of *N. fowleri* in a plate	77000	82000	85000	79500	82000
Bacterial colonies diluted 0-fold	0	0	0	0	0
Total bacterial colonies grown	0	0	0	0	0

Fig. 2. Interactions between *N. fowleri* and MRSA by GlcNAc and galactose. All sequences from A1 to F2 were the same as those described in Figure 1. An asterisk (*) represented $P<0.005$. Other asterisks (**) showed the result of treating monosaccharides in co-cultured state of *N. fowleri* and MRSA.

mental results explained that GlcNAc or galactose was treated in a cultured state of *N. fowleri* and bacteria. This meant that *N. fowleri* and bacteria competitively bound to GlcNAc or galactose, since the interaction results were hardly different from those of the control group.

Through the results of this study, it was thought that the

effect of monosaccharides on the interaction of several Gram-negative or positive bacteria and the evidence that the interaction could be enhanced by longer pre-incubation time.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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