ARTICLE

Influence of *Blastocystis* on the Growth of a Representative Gut Bacterium

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ABSTRACT

Blastocystis is a commonly encountered single-celled eukaryotic protozoan found in both humans and animals. This protozoan is historically classified as a parasite as it is sometimes encountered in patients with dysbiosis. Although this association may be true, healthy individuals harboring *Blastocystis* often have no symptoms of dysbiosis and are even observed to carry more diverse gut microbiota. This study aimed to explore the growth interactions of specific *Blastocystis* subtypes (ST), namely ST1, ST2, ST3, and ST7, with a representative gut bacterium, *Escherichia coli*, by means of co-incubation and co-culturing. *Blastocystis* growth was monitored by obtaining cell counts through hemocytometry where co-incubated cultures were compared against singly incubated *Blastocystis* cells. *E. coli* growth was monitored using the drop plate assay to obtain a large number of observed colony forming units (CFU). Results from the experiments show that ST2 and ST3 significantly increased in number after co-incubation ($P = 0.0123$, $P = 0.0279$, respectively). It was also observed that the number of CFU produced by *E. coli* significantly increased when co-cultured with all tested *Blastocystis* subtypes ($P = 0.0300$, $P = 0.0080$, $P = 0.0076$, $P = 0.0002$ for ST1, 2, 3, and 7, respectively). The findings of this study may be used to investigate mechanisms of the observed microbial interactions and to understand the significance of eukaryotic gut microbiota.

INTRODUCTION

Blastocystis is an anaerobic eukaryotic organism with 17 different subtypes (ST), all of which are classified as Stramenopiles, despite having no visible flagella present in its morphology (Clark et al. 2013; Adao and Rivera 2018). *Blastocystis* is also known to be the most frequent single-celled eukaryotic organism that inhabits the mammalian gastrointestinal tract through transmission of the fecal-oral route (Audebert et al. 2016).

Difficulties on its relationship with a host organism are encountered when studies regarding its possible pathogenicity

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are discussed. Several studies that involve the metagenomic gene sequencing and bioinformatics analysis of the gut microbiota in humans conclude the positive association of *Blastocystis* in healthy individuals with diverse presence of gut bacteria compared to individuals with compromised diversity of gut bacteria due to diseases that cause dysbiosis such as irritable bowel syndrome (IBS) (Andersen et al. 2015; Audebert et al. 2016, Beghini et al. 2017; Kodio et al. 2019). However, test subject analyses of *Blastocystis* infection from other studies conclude that there is a positive correlation between IBS and an increase of *Blastocystis* infection (Jimenez-Gonzalez et al. 2011). A similar study focusing on fecal analysis of IBS diagnosed subjects also associates an inverse correlation with *Blastocystis* infection and the presence of gut bacteria (Nourrisson et al. 2014). However, the studies mentioned above involve analyses of *Blastocystis* with considerations on the specific subtypes only occurring after data processing. As such, there is a need to elucidate if the specific characteristics of different *Blastocystis* subtypes could possibly influence the activity and persistence of gut bacteria (Yason et al. 2019).

This study aimed to determine through quantitative analysis the influence of different *Blastocystis* subtypes (ST1–3,7) on the growth rates of *Escherichia coli*, a representative gut bacterium. The findings of this study could suggest possible relationships between *Blastocystis* subtypes and *E. coli* based on differences in growth outcomes.

MATERIALS AND METHODS

Blastocystis cultures

Blastocystis xenic cultures of ST1, ST2, ST3, and ST7 were provided by the Pathogen-Host-Environment Interactions Research Laboratory (PHEIRL), Institute of Biology, College of Science, University of the Philippines Diliman. *Blastocystis* ST1, ST2 and ST3 were obtained from sewage water samples while ST7 were obtained from chicken stool samples. *Blastocystis* cells were maintained and cultured in biphasic medium containing 1.5% non-nutrient agar slants overlaid with 3 mL broth containing 300 µL bovine serum heat inactivated at 56 ºC and 30 µL penicillin-streptomycin antibiotics (Rivera 2008). Cultures were maintained in tightly capped tubes and placed at 37 °C incubator. Subculturing was performed at least twice a week (Belleza et al. 2015).

Escherichia coli cultures

Escherichia coli ATCC 25922 second passage culture was provided by PHEIRL. The isolate was maintained in Luria-Bertani medium (both broth and agar form) at 37 °C.

Co-culture experiments

Viable *Blastocystis* cells were isolated by sucrose-suspension. In one microcentrifuge tube, 1 mL of *Blastocystis* cell culture was centrifuged for 1,000 rpm for 2 min using a microcentrifuge, decanted, and resuspended in 700 µL sterilized distilled water. Additional 700 µL of freshly prepared 3.11 M sucrose suspension or an estimated specific gravity of 1.066 was poured gently to promote layer formation (Hoevers et al. 2000). The tube containing *Blastocystis* cells and sucrose suspension was centrifuged at 4,200 rpm for 10 min in a temperature of 10 ºC. From the formed layers, 600 µL of the clean upper cell suspension overlay was transferred to a clean microcentrifuge tube. The newly transferred *Blastocystis* cell suspension was then centrifuged at 10,000 rpm for 2 min in a temperature of 20 ºC, decanted, and resuspended in 1 mL sterilized distilled water. Cell concentration in cells/mL was quantified using a hemocytometer.

Select concentration of 24-h old *E. coli* was quantified by using a 1.0 McFarland standard and suspended in 1X phosphate-buffered saline (PBS).

Both *Blastocystis* and *E. coli* cells were washed twice with 1X PBS and centrifuged at 1000 x g for 10 min. A concentration of 1 x 10^5 cells/ml of *Blastocystis* and 1 x 10^8 cells/ml of *E. coli* was incubated in pre-reduced PBS for 24 h at 37 °C. A previous study used a 1:100 ratio of *Blastocystis*:bacteria ratio (Yason et al. 2019). This study made use of 1:1000 ratio to check if growth changes can also be observed even if the number of *Blastocystis* is more reduced. Controls with only 1 x 10^5 cells/ml of each *Blastocystis* ST and 1 x 10^8 cells/ml of *E. coli* were similarly prepared.

After a 24-h incubation period, *Blastocystis* cell concentration was quantified with a hemocytometer. This was repeated for three trials per set-up, with three replicates per trial.

A drop plate assay adapted from Herigstad et al. (2001) was performed to determine the number of bacterial colony-forming units (CFU) produced per set-up. For each of the three trials, duplicate plates were produced per set-up. Per co-culture set-up, three to five-fold dilutions were prepared. Four-10 µL drops of each dilution of each culture set-up were placed with sufficient spacing and laid out in a gradient manner. Drops on the plate were allowed to dry before incubation at 37 °C for 24 h.

Data analysis

Shapiro-Wilk test was used to determine the normality of gathered data. Comparisons between the CFU of *Blastocystis* and *E. coli* in the control and experimental co-cultures were done using Welch's *t*-test for paired samples with a 95% confidence interval. Statistical analysis and graph generation was performed using Prism GraphPad version 8.0.2.

Photographs of microbial growth and other miscellaneous photos were digitally enhanced with paint.net version 4.2.10.

RESULTS

Blastocystis cell cultures

Growth of *Blastocystis* cells appeared to be limited on solid substrate as cells were observed to aggregate on the surface of the solid agar in the biphasic culture medium particularly at the bottom of the slant. As for each subtype, growth was present in all cultures, with additional film observed in ST3 after incubation. All *Blastocystis* cells that were used for experimentation, however, were taken from the clumped cell aggregate at the bottom of the agar slant.

Blastocystis cells consistently appear to be globular and devoid of any visible vacuole (Figure 1). Regardless of subtype, all *Blastocystis* cells were less than 2 µm in diameter. The largest cells were observed from cultures of ST2.

E. coli positively affected the *Blastocystis* cell counts

E. coli was co-cultured with *Blastocystis* in a nutrient-free medium. Different subtypes of *Blastocystis* were co-incubated with *E. coli* and compared against *Blastocystis* ST1, ST2, ST3, and ST7 that were incubated without the bacteria. All subtypes exhibited increased cell count compared to their respective control set-up. As shown in Figure 2, statistically significant increase in *Blastocystis* cell count was observed in *E. coli* co-incubations with ST2 ($P = 0.0123$) and ST3 ($P = 0.0279$).

Blastocystis positively affected the growth of *E. coli*

The CFU counts of *E. coli* co-cultured with different *Blastocystis* subtypes were determined and examined by using

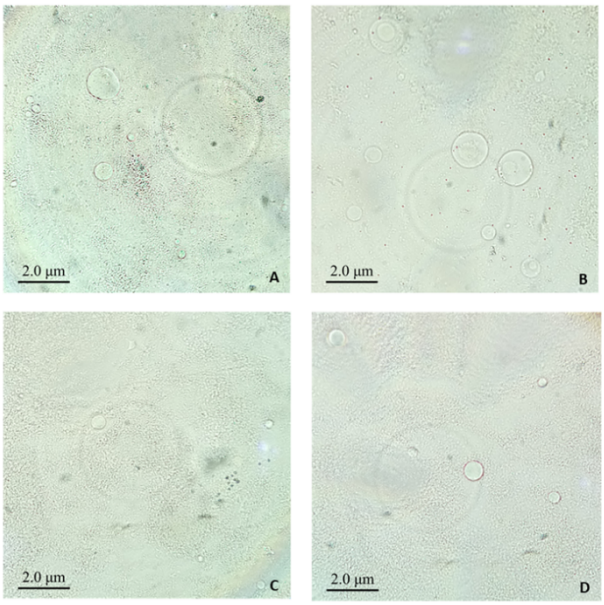

Figure 1: *Blastocystis* cells observed at 1000x. All cells observed in the figures were obtained from pure cultures. Photomicrographs A, B, C, and D refer to *Blastocystis* ST1, ST2, ST3, and ST7, respectively. Regardless of size, all cells have similar shape and appearance.

the drop plate method. Shown in Figure 3 are representative images of *E. coli* growth when individually co-cultured with select *Blastocystis* subtypes. All drop plating resulted in round, whitish colonies with growth limited to the area on which the applied drop evaporated. Generally, *E. coli* is observed to have increased growth when co-cultured with *Blastocystis*, regardless of subtype. This is particularly observed at 10^{-5} dilution wherein the *E. coli* cultures cultivated with different *Blastocystis* subtypes had more colony counts than the control.

However, differences between the number of colonies can be observed from different co-culture set-ups with different *Blastocystis* ST involved. Visually in Figure 3, ST2 had the highest positive influence on bacterial growth as *E. coli* colonies appeared to be confluent despite the decreasing dilutions applied for each successive drop. This was followed by ST3, then ST7 and lastly ST1. The number of colonies were counted in agar plates from technical replicates and averages were calculated. These were then used to generate the graph in Figure 4. Again, as shown in Figure 3, cultivation of *E. coli* with *Blastocystis* ST2 showed the highest bacterial count. Despite having visually larger growths, no statistically significant effect was established with *p* values of 0.0080 and 0.0076 for co-cultures of *E. coli* and ST2 and *E. coli* and ST3, respectively. For co-cultures of *E. coli* and ST1 and *E. coli* and ST7, decreasing dilutions appeared to have a more significant effect on the number of *E. coli* colonies formed, with a reduction in colonies as dilutions were increased. This, however, is appears to be statistically significant only for co-culture of *E. coli* and ST7 with *p* value of 0.0002.

DISCUSSION

This study is the first to investigate the growth rate effects of *Blastocystis* ST1 and ST2, and *E. coli* in culture conditions. Results suggest that *Blastocystis* ST2 and ST3 may be symbiotic with *E. coli* since the protozoan was able to proliferate while *E. coli* increased in growth when grown in co-culture conditions. However, ST1 and ST7 might be exhibiting a commensal relationship as growth was only observed in *E. coli*. Currently, the identity of *Blastocystis* as a parasite or commensal organism has yet to be established due to conflicting studies regarding its

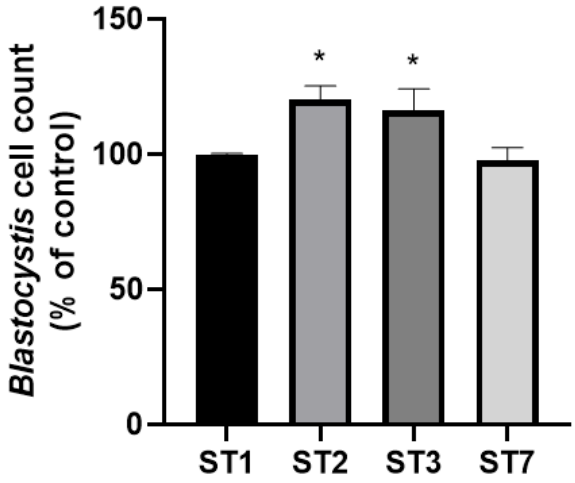

Figure 2: Changes in cell counts of various *Blastocystis* subtypes when co-incubated with *E. coli* in PBS for 24 h at 37 ºC. All subtypes had a higher growth compared to their respective controls. Significant differences in growth are observed in ST2 and ST3. Single asterisks signify $p < 0.05$. Alpha = 0.05.

association with its host organism. Studies that describe *Blastocystis* as a parasite often relate its prevalence with gastrointestinal disease such as diarrhea and abdominal pain (Tan 2008). Studies such as fecal analysis of patients suffering from IBS revealed that there is a positive correlation between a decrease in gut bacteria, specifically *Bifidobacterium* sp., and *Blastocystis* colonization (Nourrisson et al. 2014). Although the aforementioned meta-analytical study mentions that there are no relations between specific *Blastocystis* subtypes and pathogenic potential, a study focusing on *in vitro* and *in vivo* testing of ST7, a specific *Blastocystis* subtype with characteristics described to have strong links with gastrointestinal symptoms, revealed a decrease in *Bifidobacterium* and both *Bifidobacterium* and *Lactobacillus*, respectively (Yason et al. 2019).

However, several studies also describe the neutral and possibly positive interactions between *Blastocystis* and gut disease. An early study that compares *Blastocystis* with both symptomatic and asymptomatic patients shows that although a higher concentration was seen in the former group, no significant differences between the prevalence of the protozoan is found between the test groups (Udkow and Markell 1993). Recently, a study concerned with elucidating whether the changes in the gut bacterial microbiome of asymptomatic patients are caused by *Blastocystis* or symptoms due to its colonization shows an increase of bacterial diversity is indeed associated with *Blastocystis* colonization (Nieves-Ramírez et al. 2018). Similar results are also obtained when comparisons between *Blastocystis* infected and uninfected subjects, suggesting the commensal behavior of the protozoan (Audebert et al. 2016).

A similar study by Lepczyńska and Dzika (2019) utilizing both xenic and axenic cultures of *Blastocystis* ST3 with *Lactobacillus lactis* and *L. rhamnosus* showed an inhibition of the protozoan's growth while *Enterococcus faecium* and *E. coli* initially promoted but eventually inhibited the protozoan's growth, specifically peaking after four days of co-incubation with *E. coli*. It is suggested that after phagocytosis, amoebic *Blastocystis* may be negatively affected as endotoxins such as lipopolysaccharides produced by *E. coli* would damage the protozoan. This was not observed in the present study as the protozoan and bacteria were only co-incubated for only 24 h before subsequent data gathering. However, this does not limit the fact that the observed vacuolar morphological form of *Blastocystis* may exhibit an outer slime coat which can also be used to trap bacteria for

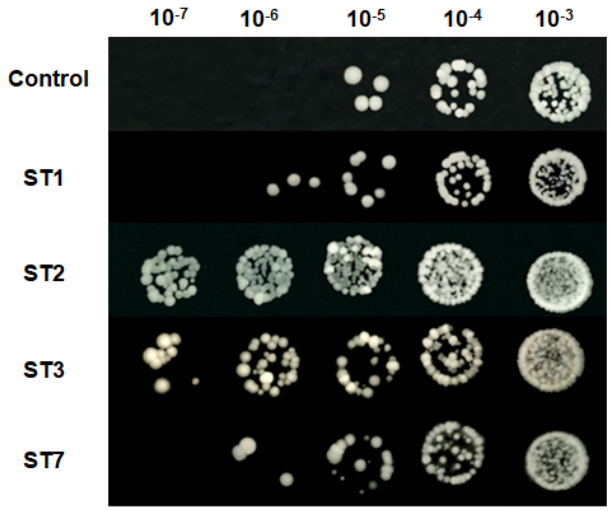

Figure 3: Growth of co-cultured *E. coli* on nutrient agar (NA) after co-incubated with *Blastocystis* in PBS for 24 h at 37 ºC. Drop plating method was used to limit the area of growth of *E. coli* colonies. From the left to right columns, drops applied on NA have decreased dilutions from the original co-culture, as indicated by the numbers found above each column.

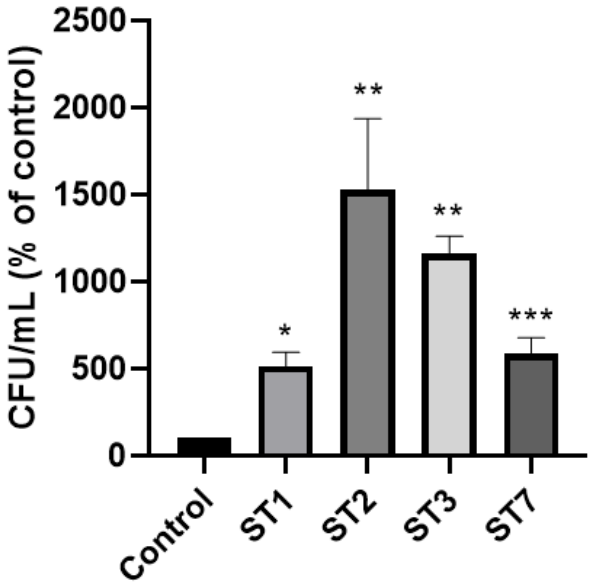

Figure 4: Differences in growth of co-cultured *E. coli* as compared to pure cultures. Data and analysis taken were limited to drops that had a 10^{-5} dilution of the original co-culture. Compared to the pure control culture, all co-cultures had a higher growth of 500 percent. All observed growth for each set-up differed significantly from the control set-up, as denoted by the asterisks. Single asterisks signify $p < 0.05$, double asterisks signify $p < 0.01$, triple asterisks signify $p < 0.001$. Alpha = 0.05.

nutrition (Zaman et al. 1998; Tan et al. 2008; Jeremiah and Parija 2013; Adao and Rivera 2018). Besides the temporal limitations of the present study, it is possible that this factor is dependent on the *Blastocystis* subtype as significant *Blastocystis* growth was only observed in ST2 and ST3.

Positive growth of *E. coli* when co-incubated with *Blastocystis* coincides with metagenomic studies suggesting that gut microbiota diversity increases (Andersen et al. 2015; Audebert et al. 2016, Beghini et al. 2017; Kodio et al. 2019). Although the present study demonstrates that microbial growth of *E. coli* increases, no study specifies any underlying factors that *Blastocystis* cells may have to influence the specific growth of the bacteria. During enteric colonization, *Blastocystis* releases IgA proteases to counter IgA release of the body's defense mechanisms, leading to more effective colonization of the protozoan and other bacteria (Puthia et al. 2005). Results from the present study, however, suggest that other possible mechanisms independent of proteases support *Blastocystis* and bacterial growth since the body's natural response to the protozoan was not simulated in any of the set-ups. Studies focusing on any underlying mechanisms of *Blastocystis* physiology are needed to understand how the protozoan benefits *E. coli* growth.

Differences in ATP generation may have also influenced growth between *Blastocystis* and *E. coli* since the former is an obligate anaerobic protozoan whereas the latter is facultative anaerobic bacteria (Clark et al. 2013; Shewaramani et al. 2017; Adao and Rivera, 2018). More numerous *E. coli* growth with *Blastocystis* may be attributed to an increased rate of mutation, and therefore survival, due to anaerobic conditions, but the chances of so are extremely slim since mutations have only been observed in long-term experiments (Shewaramani et al., 2017). It may have been possible that during incubation, *E. coli* may have removed the majority of oxygen present in its culture media, improving both growth of the bacteria and *Blastocystis*. This does not explain, however, why *E. coli* was able to grow more favorably when cultured with *Blastocystis* as the protozoan has no known bearing in improving conditions for *E. coli* growth. More research focusing on this aspect is needed.

To further understand the influence of *Blastocystis* on *E. coli* growth, the energy utilization of *E. coli* may be monitored through tracking carbon utilization. Under specific conditions wherein carbon is readily available while other nutrients are limiting, *E. coli*, like other bacteria, synthesizes glycogen and utilizes it as a compound for energy storage for prolonged survivability (Jones et al. 2008). In order to degrade and catabolize synthesized glycogen in carbon deficient conditions, *E. coli* produces GlgX proteins to promote the shortening of glycogen chains (Dauvillée et al. 2005). It has been suggested by a previous study that expression of the *glgX* gene can be used as an indicator of proper gut colonization by *E. coli* as mice with *glgP* mutants, wherein GlgP is a protein needed for glycogen phosphorylation for GlgX activity to proceed, proved to have defective colonies found in the intestines (Jones et al. 2008). With a better understanding of *E. coli* carbon utilization, implications on how the bacterium responds to its environment when co-incubated with *Blastocystis* can be made.

The findings of this study suggest possible relationships between *E. coli* and several *Blastocystis* subtypes. *Blastocystis* exhibited subtype-dependent differential growth responses after co-incubation with *E. coli*. Its growth was significantly higher after co-incubation with any of the tested *Blastocystis* subtypes. These results suggest that certain subtypes of *Blastocystis* do increase gut microbiota while also limiting their own proliferation when exposed to the same environment, preventing any possible enteric conditions or diseases caused by the protozoan itself, if any. This study is the first to report on the interactions of ST1–3 with a common gut bacterium. These STs are considered commensal but now may be considered indirectly harmful when they cause *E. coli* to increase to the potential detriment of the other members of the gut microbiota. Due to limitations of the experiment and the current pandemic, no further investigations were done to determine the underlying mechanisms for the suggested microbial relationships.

It is recommended for future studies to use genetic analysis for data interpretation. Carbon utilization of *E. coli* may be monitored to obtain any possible explanations on how *Blastocystis* may affect energy utilization of *E. coli.* The expression of *glgX* may be monitored given its role in glycogen utilization in carbon-limiting environments.

To obtain more accurate data, quantification of both *Blastocystis* and *E. coli* by flow cytometry and quantitative polymerase chain reaction (qPCR), respectively, may be done following the procedures of Yason et al. (2019).

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