Escherichia coli Nissle 1917 forms biofilm and outgrows *Escherichia coli* K12 in a temperature-dependent manner

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SUMMARY Planktonic *Escherichia coli* may grow as biofilms adhered to a surface when environmental stressors are detected. Previous studies utilizing co-culture (culturing of two or more cell populations in contact with one another) and dual-species biofilm-based methods have found that probiotic *E. coli* Nissle 1917 (EcN) may inhibit biofilm formation of other *E. coli* strains and outcompete their growth. However, the exact mechanism of biofilm inhibition has yet to be elucidated. Using the EcN and *E. coli* K12 MG1655 (K12) strains, we investigated biofilm inhibition using a biofilm quantification assay. Growth curves for EcN and K12 were compared at 30°C and 37°C. Subsequently, biofilm formation assays were performed according to a previously established protocol at 30°C and 37°C. Growth curve analysis showed two exponential growth phases for EcN at 30°C (n=3), with the strain reaching a higher final OD_{600} compared to K12. At 37°C, K12 underwent two exponential growth phases, and the strains grew to similar OD_{600} values after 24 h. Consistent with the growth curves, EcN formed significantly more biofilm than K12 at 30°C (p=0.0012). Neither strain formed quantifiable biofilm at 37°C. Given higher rates of biofilm formation in EcN than K12, we infer that in co-culture, the majority of the biofilm quantified would consist of EcN rather than K12. Our current protocol appears unsuited for assaying biofilm inhibition in co-cultures. Future studies may instead use supernatant-based assay methods to determine whether EcN secretions affect K12 biofilm formation.

INTRODUCTION

*E*scherichia coli (*E. coli*) can exist as either free-swimming bacteria or a community of sessile (non-motile) bacteria adhered to a surface (1). These communities, known as biofilm, are often involved in chronic infections and are difficult to eliminate due to their high tolerance to environmental stresses (1). Motile bacteria transition to form biofilm when there is a change detected in the environment, such as a change in nutrient availability (3). Biofilm formation can also be stimulated or inhibited by the presence of other bacterial species in the environment, and biofilms themselves can include multiple species of bacteria (1). Sessile bacteria can be up to 1000-fold more resistant to antibiotics and can endure host immune defense responses, raising concerns in health settings (2). Understanding biofilm formation is therefore critical for handling infections.

E. coli Nissle 1917 (EcN) is a probiotic strain with clinical applications in human and veterinary intestinal disorders such as inflammatory bowel disease (1). When present on the surface of the intestinal lumen, EcN is capable of improving host immune response and inhibiting biofilm formation of other *E. coli* strains (1). Previous experiments have also shown that EcN is capable of inhibiting enterohemorrhagic *E. coli* (EHEC), *S. aureus,* and *S. epidermidis* in co-culture (1). A co-culture is an experimental set-up in which two or more populations of cells are grown in contact with each other (4). For bacterial co-cultures, the populations are usually grown on an agar plate or in nutrient broth together. The mechanism responsible for biofilm inhibition by EcN in co-culture is still poorly understood. Previous studies have shown that DegP, a protease and chaperone protein secreted by EcN, is responsible for repressing biofilms in other bacterial species (1). It has been suggested that secreted DegP may interact with the Cpx biofilm formation pathway in other bacteria, but the exact mechanism has yet to be elucidated (1).

A preliminary study found that EcN may also be capable of inhibiting the biofilm formation of *E. coli* K12 MG1655 in co-culture (5). However, the findings were not

conclusive due to large deviations between treatments. Using the same strains, we investigated biofilm inhibition in co-cultures using a biofilm quantification assay and hypothesized that co-culture with EcN would decrease biofilm formation of the *E. coli* K12 strain.

METHODS AND MATERIALS

Bacterial strains and growth conditions. *E. coli* K12 MG1655 (K12) and *E. coli* Nissle 1917 (EcN) were obtained from the Microbiology and Immunology department at the University of British Columbia. Both strains were grown on Lysogeny Broth (LB) agar at 37°C. After incubation for 16 to 18 hours, plates were either immediately used for colony selection or kept refrigerated at 4°C for future use. Plates stored at 4°C for longer than 2 weeks were re-streaked onto a fresh plate before use. Overnight liquid cultures were prepared by inoculating refrigerated colonies into 5 mL of LB and incubating at 37°C for 16 to 18 hours with shaking.

Bacterial strain verification. A genomic miniprep was performed on overnight cultures of each bacterial strain using the PureLink^{TM} Genomic DNA Mini Kit (Invitrogen^{TM}). A260/280 ratios were obtained using a NanoDrop spectrophotometer (Nanodrop 2000c Spectrophotometer; ThermoScientific) and the genomic DNA was subsequently sequenced by GENEWIZ, Inc.

Growth curve comparison. Overnight cultures of the K12 and EcN strains were diluted to achieve a starting OD_{600} value of approximately 0.02. 200 µL of the diluted cultures were transferred to a flat-bottom 96-well plate in triplicate. 200 µL of LB was used as the negative control (in triplicate). Plates were incubated at 30°C or 37°C with shaking at 560 rpm for 3 seconds every 10 minutes in an automatic microplate reader (Synergy H1 Hybrid Multi-Mode Reader; BioTek). OD_{600} readings were measured every 10 minutes over a 24-hour period.

Biofilm formation assay in glass test tubes. To generate monocultures of each strain, plate-isolated colonies were inoculated into 1 mL LB in loosely-capped glass test tubes by gently touching a sterile pipette tip to a colony. To set up the assay in triplicate, the same isolated colony was reused for subsequent inoculations. An LB-only tube served as a negative control. The cultures were incubated at 30°C or 37°C without shaking for 24, 48, or 72 hours. After incubation, the culture media was gently removed using a micropipette, being careful not to disturb the ring of biofilm adhered to the glass tube. Tubes were then washed 3 times using dH_2O and dried overnight upside down.

Crystal violet staining of biofilm. The biofilm ring was stained by pipetting 2 mL of 0.1% crystal violet solution into each tube and incubating for 15 minutes at room temperature (RT). The staining solution was then removed and tubes were washed 5 times with dH_2O. Tubes were then dried upside down overnight. To quantify the biofilm on the tube, the crystal violet was eluted with 2 mL of 95% ethanol for 30 minutes at room temperature. 200 µL of eluted solution was transferred to a flat-bottom 96-well plate for an A_{590} reading on

Table 1. Biofilm formation assay conditions across studies.

Study	Temperature	Formation Vessel	Incubation Time
Current Study	30°C	New glass tubes	24 h
Fung *et al.* (in press)	30°C	Glass tubes	72 h
Fang *et al.* (Scientific Reports)	37°C	Polypropylene tubes	24 h

the spectrophotometer (Epoch; BioTek). 95% ethanol was used as the blank.

Statistical analysis. Statistical significance was determined using two-tailed *t*-tests assuming equal variance between K12 and EcN strains. The null hypothesis was that the mean biofilm formations of the two strains are equal, and significance was defined as having a p-value less than 0.05. One star on a figure denotes a p-value less than 0.05 and two stars denote a p-value less than 0.01.

RESULTS

Validation of *E. coli* K12 and EcN strains. To confirm the identity of each strain, genomic DNA was extracted and sent for 16S gene sequencing. A BLAST search indicated that the raw K12 sequence (6) aligned with the published sequence at 99.32% similarity, and the raw EcN sequence (7) aligned with the published sequenced at 99.66% similarity. These results confirm the expected identity of our working *E. coli* strains.

Growth curves of *E. coli* K12 and EcN strains are temperature-dependent. Next, we assessed whether the growth phases of K12 and EcN are comparable and suitable for co-culture by performing growth curve analyses on monocultures of each strain. Preliminary work suggests that biofilm formation in glass test tubes may be greatest at 30°C (5). Therefore, for each strain, we conducted growth curve analyses at 30°C and 37°C to assess differences in growth due to temperature. OD_{600} readings were collected every 10 minutes over a 24 hour period from overnight monocultures that were diluted in LB. Figures 1 and 2 show the growth phases of K12 and EcN at 30°C and 37°C, respectively. At 30°C, EcN undergoes two exponential phases between the first 1-5 hours of growth and 12-18 hours of growth, while K12 undergoes one growth phase and consistently produces lower OD values than EcN (Fig. 1). At 37°C, the opposite is observed as K12 appears to undergo two exponential phases between 1-2 hours and 7-10 hours of growth. Both strains appear to plateau at similar OD values after 24 hours of growth (Fig. 2). The LB-only negative control shows no detectable change in OD over the time period. Altogether, these results indicate that the growth phases of K12 and EcN may be temperature-dependent.

EcN strain forms more biofilm than K12 after 48 hour and 72 hour incubation. In order to establish the baseline biofilm formation of each strain, we performed the biofilm
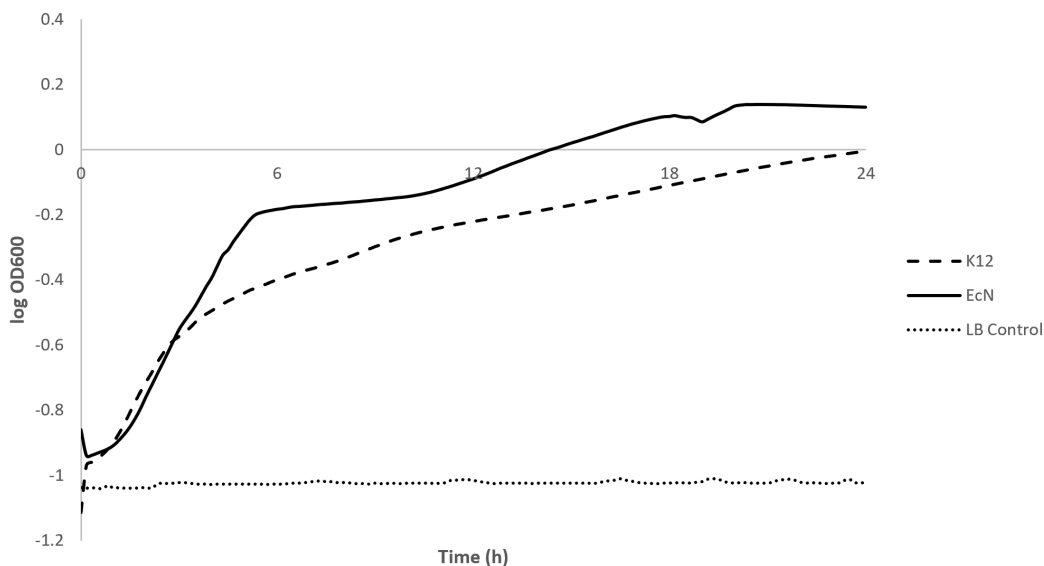
FIG. 1 *E. coli* Nissle 1917 growth curve exhibits two exponential growth phases in a 24 h incubation at 30°C. Overnight cultures of the *E. coli* EcN and K12 strains were diluted to a starting OD_{600} of approximately 0.02. Diluted cultures and a negative control of Lysogeny Broth were plated in triplicates into a 96-well plate, and incubated at 30°C using the Biotek Synergy H1 Hybrid Multi-Mode Reader. OD_{600} readings were measured with shaking at 560 RPM for 3 s every 10 m over a 24 h period to generate growth curves.

formation assay on monocultures of K12 and EcN. After incubation at 30°C for 24, 48, or 72 hours, the culture media was gently removed and the ring of biofilm adhered to the test tube wall was stained with crystal violet (Fig. 3). Finally, the crystal violet was eluted with ethanol and the amount of biofilm formed was indirectly quantified by measuring the absorbance. Figure 4 shows that EcN forms significantly more biofilm than K12 after 48 h (p = 0.018) and 72 h (p = 0.003, two-sample t test, equal variances). Additionally, K12 did not form quantifiable biofilm at 48 and 72 hours (Fig. 4). At 24 hours, no quantifiable amount of biofilm is formed by either strain; this is indicated by a higher absorbance value observed in the LB-only negative control. Together, these results demonstrate that the EcN strain forms more biofilm than K12 following a 48-hour or 72-hour incubation.

EcN strain forms more biofilm than K12 at 30°C. To investigate whether biofilm formation in monocultures is temperature-dependent, we compared biofilm formation of K12 and EcN strains at 30°C and 37°C using the biofilm formation assay. We minimized non-specific crystal violet staining by using new glass tubes to avoid staining of residual material left on the tube interior surface (Fig. S1). Figure 5 shows that after incubation for 24 hours, EcN forms more biofilm than K12 at 30°C (p = 0.0012). At 37°C, neither strain formed quantifiable biofilm. Although using new glass test tubes results in tighter error bars in all conditions, the LB-only negative control still yielded high absorbance readings. Consistent with previous work, these results indicate that EcN forms more biofilm than K12 at 30°C, suggesting that biofilm formation may be temperature-dependent.

DISCUSSION

Biofilm formation of pathogenic bacteria play a role in the persistence of human disease. The probiotic *E. coli* Nissle 1917 strain naturally colonizes in the human gut and has been shown to inhibit biofilm formation of other bacterial strains (1). However, the EcN mechanism of inhibition is poorly understood. In this study, we investigated biofilm inhibition in EcN and K12.

EcN and K12 growth rates are temperature-dependent. The results show that EcN forms significantly more biofilm than K12 and undergoes double-exponential phase growth
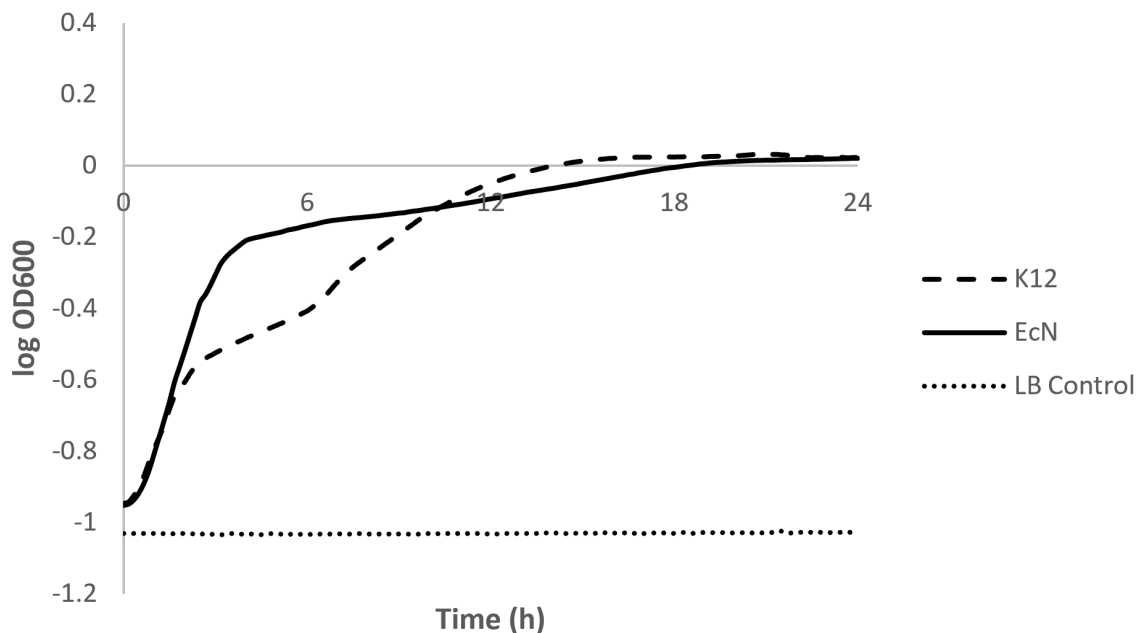

FIG. 2 *E. coli* K12 growth curve exhibits two exponential growth phases in a 24 h incubation at 37°C. Overnight cultures of the *E. coli* EcN and K12 strains were diluted to a starting OD$_{600}$ of approximately 0.02. Diluted cultures and a negative control of Lysogeny Broth were plated in triplicates into a 96-well plate, and incubated at 37°C using the Biotek Synergy H1 Hybrid Multi-Mode Reader. OD$_{600}$ readings were measured with shaking at 560 RPM for 3 s every 10 min over a 24 h period to generate growth curves.

at 30°C but not at 37°C. This suggests that biofilm formation and rate of growth for EcN are both temperature-dependent. However, the 37°C growth curve was only performed once and should therefore be repeated to yield more conclusive evidence. It is also possible that the EcN strain's increased biofilm formation at 30°C is due to its increased growth rate. However, previous studies have found that there is no correlation between the capacity to form a good biofilm and fast growth (8). Instead, it has been suggested that EcN is an inherently dominant biofilm producer, but responsible genes remain to be determined (8).

At 30°C, the two exponential phases of EcN occur in the first 1-5 hours of growth and between 12-18 hours of growth, as seen in Figure 1. This effect is not observed in K12 at 30°C, but it is seen at 37°C (Fig. 2). The double exponential growth phases of EcN and/or K12 may be due to diauxic (or biphasic) growth. Diauxic growth can be observed when bacteria are grown on media that contains two sugars, with the two growth stages separated by a lag phase indicated by arrested growth (9). This is usually an adaptation to maximize population growth in environmental conditions with multiple nutrients (9). EcN and K12 were grown in LB, which contains catabolizable amino acids as carbon sources as opposed to sugars (10). Diauxic growth has been observed in K12 due to the use of certain carbon sources followed by a metabolic switch to use another, with data showing a sequential catabolism of amino acids (10). The amino acid composition of LB has also been shown to vary with age, time of autoclaving, and between batches (10). The 37ºC growth curve showing double exponential growth for K12 was only done once, however, and needs to be replicated for more confidence in the result.

A recent study has shown that EcN undergoes diauxic growth under conditions when different nutrient metabolism pathways are advantageous (11). The study also demonstrated that stress responses in low-diversity conditions can cause genomic instability, and that EcN adaptation is driven by carbohydrate availability (11). EcN is a more genetically complex strain than K12, given that EcN is a probiotic found in humans and K12 is a commonly used lab strain, which has likely lost genetic diversity. It is possible that EcN optimally expresses a protein or factor at 30°C (or this protein is more active at 30°C) that K12 does not, allowing it to utilize a different carbon source and achieve diauxic growth. Further work needs to be done to elucidate the mechanism of the double exponential phase growth exhibited by EcN and to determine if there is a difference in nutrient availability at different time-points in the growth curve.

Biofilm formation conditions. The biofilm formation assay conducted in this study was performed in clean glass tubes at 30°C. Table 1 compares this method to other published methods of biofilm quantification. Our results show that EcN forms significantly more
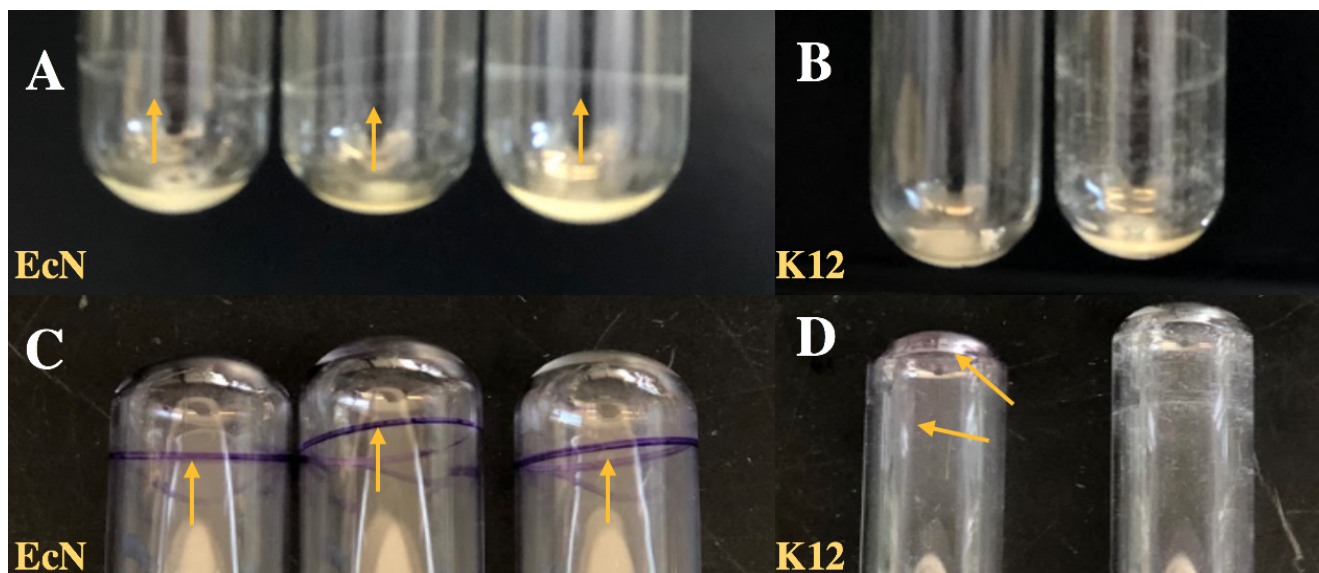

FIG. 3 Biofilm rings observed after a 72 h incubation and subsequent crystal violet staining. Yellow arrows indicate the location of biofilm rings observed after K12 and EcN monocultures were removed and tubes were washed with dH₂O (A, B), and after staining with 0.1% crystal violet (C, D).

biofilm than K12 (p = 0.0012), which does not appear to form quantifiable biofilm. This is contrary to a previous study, which showed limited EcN biofilm production using a similar assay (5). However, their study did not use new tubes and had large deviation between treatments, which may have contributed to the discrepancy between their results and ours (5). It has been established that EcN readily forms biofilm on abiotic surfaces and can outcompete other bacteria with its own biofilm (8). The study by Fang *et al.* also showed substantial biofilm formation by EcN; however this was shown at 37ºC in polypropylene tubes (1). Both studies referenced in Table 1 displayed K12 biofilm growth, which our study was unable to produce. Further comparisons and optimizations of biofilm formation assays could elucidate why the assay described in our study did not result in K12 biofilm formation.

Limitations of the assay. Prior to co-culture experiments, biofilm formation assays were performed to determine baseline biofilm formation of monocultures of K12 and EcN. Challenges were encountered in producing reliable and consistent results. Neither strain produced quantifiable biofilm after a 24-hour incubation, which was indicated by absorbance readings lesser than that of the LB-only negative control. We predicted both strains to have an increased biofilm formation at longer incubation periods since biofilm matrix forms and matures overtime. However, unlike EcN, K12 still did not form quantifiable biofilm after 48 and 72 hours.

Since absorbance readings for the negative control and working strains greatly varied between replicates, we hypothesized that the pre-used glass tubes being utilized were not thoroughly cleaned and that leftover residue in the tube was being stained aside from the K12 and EcN biofilms, thus implicating our results. To minimize non-specific crystal violet staining, new glass tubes were used for another biofilm formation assay at a 24-hour incubation. More consistent results between biological replicates were generated and, from this, we concluded that K12 does not form quantifiable biofilm under the conditions of our current method. The lack of biofilm detected in K12 contradicts a previous study (5), in which strongly stained biofilm rings were observed for K12 cultures under the same conditions. Overall, our results suggest that EcN forms significantly more biofilm than K12,
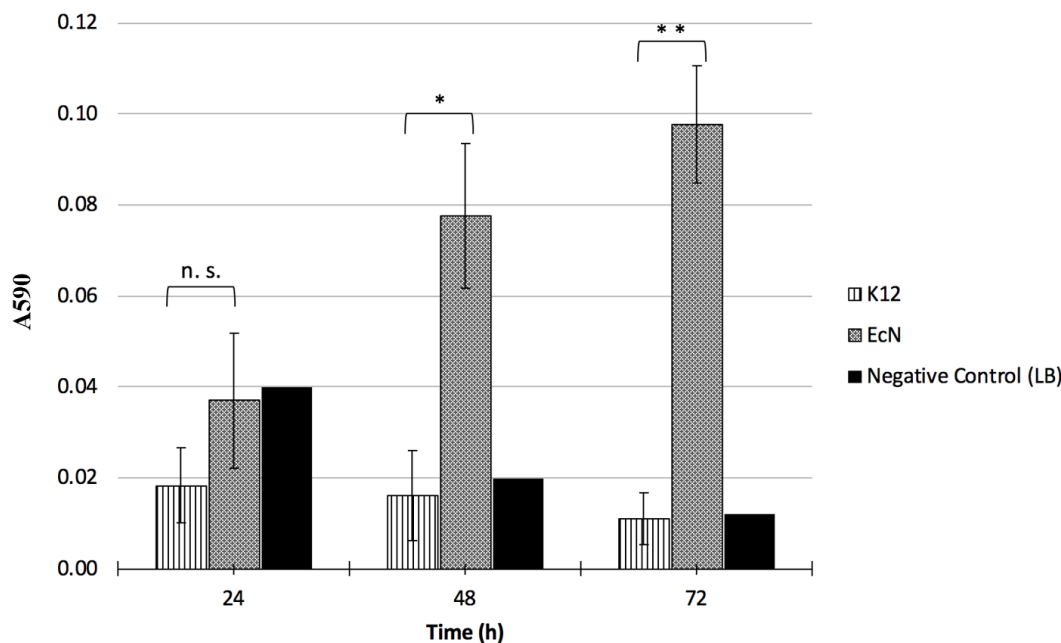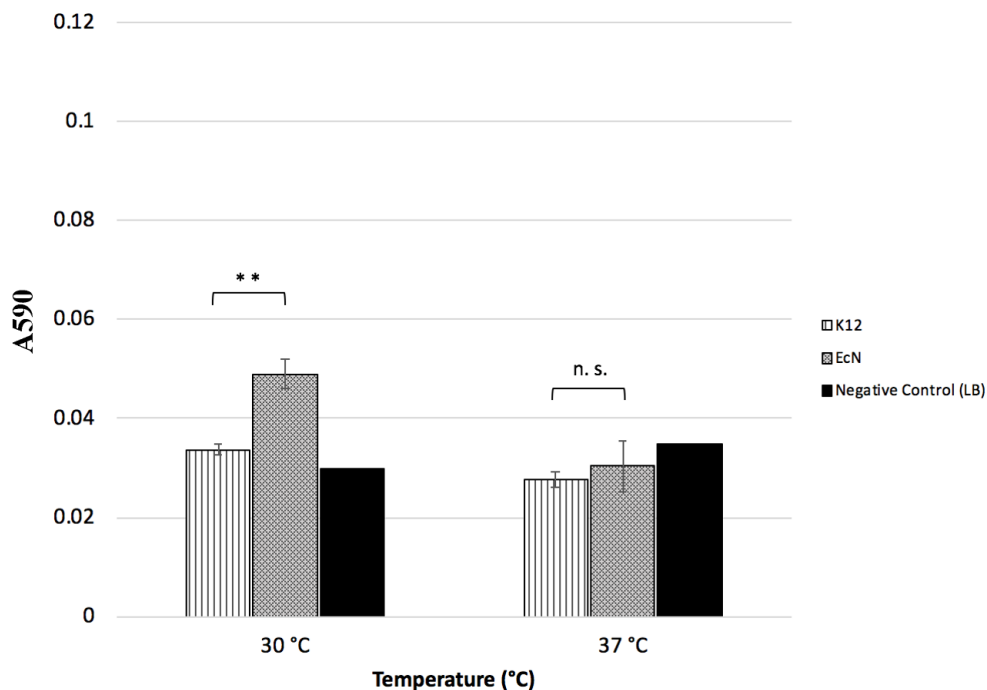

FIG. 4 *E. coli* Nissle 1917 forms biofilm at 48 h and 72 h. Plate-isolated colonies of K12 (n=2) and EcN (n=3) were inoculated in 1 mL of LB in loosely-capped test tubes. The monocultures and an LB-only control were incubated at 30ºC without shaking for 24, 48, and 72 h. Culture media was then removed and biofilms were stained with 0.1% crystal violet solution. The crystal violet stain was eluted with 95% ethanol and samples were plated into a 96-well plate. A_{590} readings were measured using the Biotek Synergy H1 Hybrid Multi-Mode Reader (p-value = 0.018, 0.003).

which does not form any quantifiable biofilm. The effect of EcN biofilm inhibition cannot be assessed using a co-culture assay with K12, as the majority of biofilm observed would be that of EcN rather than K12 as previously expected. Thus, we did not proceed with co-culture experiments and recommend that different protocols be developed for testing the biofilm inhibition hypothesis.

Conclusions Our results suggest that in a liquid co-culture of EcN and K12, the majority of quantifiable biofilm is formed by the EcN strain. Since our assay cannot determine how much of the biofilm is contributed by the EcN and K12 strains respectively, the method is unsuited for studying the inhibitory role of EcN in biofilm development of K12. We also demonstrate that both the K12 and EcN strains may undergo multiple exponential growth phases in a temperature-dependent manner. This finding may provide valuable insights into the metabolic characteristics of EcN and its probiotic efficacy.

Future Directions Various aspects of the biofilm formation assay may be optimized to improve the consistency and reliability of the results. Other studies have utilized propylene culture tubes rather than glass tubes for assaying biofilm (1). Surface roughness and topography are among the characteristics of culture substrates that have been demonstrated to affect the biofilm attachment process and alter the amount of biofilm quantified (12). Thus, repeating the presented biofilm assay using different culture substrates is a key experiment in elucidating the mechanisms responsible for biofilm formation and inhibition in the EcN and K12 strains. Additionally, the crystal violet elution step of the biofilm assay may be performed using a lower elution volume of 95% ethanol. This will concentrate the reconstituted stain to yield absorbance values greater than 0.1, which is commonly accepted to be the ideal lower limit for absorbance readings.

   Further assessments of the presented biofilm assay are needed to determine the best method for studying biofilm inhibition in co-cultures and the CpxR pathway. Given that EcN appears to form significantly more biofilm than K12 at 30°C, a co-culture method may not be optimal for studying the inhibitory effects of EcN on K12. Instead, a supernatant-

FIG. 5 *E. coli* Nissle 1917 forms more biofilm at 30ºC than 37ºC. Plate-isolated colonies of K12 (n=3) and EcN (n=3) were inoculated in 1 mL of LB in loosely-capped test tubes. The monocultures and an LB-only control were incubated at 30ºC and 37ºC without shaking for 24 h. Culture media was removed biofilms were stained with 0.1% crystal violet solution. The crystal violet stain was eluted with 95% ethanol and samples were plated into a 96-well plate. A$_{590}$ readings were measured using the Biotek Synergy H1 Hybrid Multi-Mode Reader (p-value = 0.0012).

based protocol may be developed. Such a method would involve pelleting an EcN monoculture and adding the resulting supernatant to a K12 monoculture. As a preliminary study, this method would provide insights into whether a protein secreted by EcN is involved in inhibiting the K12 biofilm. Later, it could be used to investigate whether a particular protein that is isolated and purified from the supernatant (i.e. DegP) is specifically involved in the mechanism of biofilm inhibition; this would help to verify the results seen in the literature (1).

Finally, future studies may investigate the multiple exponential phases we observed in EcN and K12 growth curves. Given EcN's reliance on carbon availability in the growth medium, utilizing variations of LB with certain components removed may determine whether the strain optimally makes use of particular nutrients throughout each of its exponential growth phases at 30°C.

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CONTRIBUTIONS

Jaspreet Gill, Karen Lau, Tina Lee and Hasel Lucas contributed equally to the design and execution of all experiments and the preparation of this report.

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