Integrating the extracellular, intracellular, and intercellular pathogenic processes of the microbiome through glucose saturation, inhibition of the acetyl-CoA carboxylase subunit accA with asRNA, and through quantifying cell-to-cell quorum sensing

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Bacteria ferment the glucose, from fiber, into Short Chain Fatty Acids, which help regulate many biochemical processes and pathways. We cultured *Escherichia coli* in Luria Broth enhanced with 15mM and 5mM of glucose. The 15mM concentration of qPCR products measured , for the target gene accA was 4,210 ng/µL. The 7.5µM sample had a concentration equaled to 375 ng/µL, and the 0µM sample had an accA concentration of 196 ng/µL. The gene accA, 1 of 4 subunits for the Acetyl-CoA Carboxylase enzyme, was suppressed by asRNA, producing a qPCR concentration of 63ng/µL. Antisense RNA for accA reduced the amount of Lux-S, a vital gene needed for propagating quorum-sensing signal molecules. Our purpose was to provide a more cumulative perspective for the pathogenesis of disease within the microbiome.

Integrating the Extracellular, Intracellular, and Intercellular Pathogenic Processes of the Microbiome through Glucose Saturation, Genetic Inhibition of the Acetyl-CoA Carboxylase Subunit accA with asRNA, and through the Quantification of Bacterial Cell-to-Cell Quorum Sensing

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ABSTRACT

Bacteria ferment the glucose, from fiber, into Short Chain Fatty Acids, which help regulate many biochemical processes and pathways. We cultured Escherichia coli in Luria Broth with 15mM and 5mM concentrations of glucose. The 15mM concentration qPCR measured , for, accA was 4,210 ng/u L. The 7.5u M sample's concentration equaled 375 ng/u L, and the 0u M sample had an accA concentration of 196 ng/u L. The gene accA, 1 of 4 subunits for the Acetyl-CoA Carboxylase enzyme, was suppressed by asRNA, producing a qPCR concentration of 63ng/u L. Antisense RNA for accA reduced the amount of Lux-S, a vital gene needed for propagating quorum-sensing signal molecules. Our purpose was to provide a more cumulative perspective for pathogenesis of disease within the microbiome.

INTRODUCTION

The Western diet includes high amounts of fats, sugars, and simple carbohydrates. Due to the Western diet, diabetes, cancer, and many neurological disorders have proliferated and quickly increased the diagnosis of these diseases (1).Ingestion of dietary fiber in the US and in European diets is approximated to be more than a few grams per day. Non-digestible oligosaccharides give between 1 and 2 kcal/g of calories (1). A cause for the prevalence of diseases may be the Western diet that lacks a high source of dietary fiber. There are two main types of dietary fiber, soluble and insoluble fiber. Soluble fiber is found in fruits and vegetables while insoluble fiber includes wheat, cellulose, and inulin. Insoluble fiber is necessary because it maintains a healthy microbiome of the gut by allowing waste in the colon to become bulky for the facile removal of fecal matter from the colon. Insoluble fiber allows for the absorption of water to produce bowel movements more readily without blockage. Fiber regulates and promotes a healthy gut microbiome. A healthy microbiome has many commensal and mutual symbiotic bacterial colonies. A well-balanced microbiome prevents colon cancer by the apoptosis of cancerous cells (6). Butyrate, a SCFA, suppresses tumors because it obstructs cell propagation and induces apoptosis when added to different types of tumor cell lines (6). An important bacteria for maintaining homeostasis within the colon and digestive tract, includes E. coli, a gram-negative bacteria that resides within the large intestines (6). E. coli ferments glucose molecules into Butyrate. Butyrate is an attractive therapeutic molecule because of its wide array of biological functions, such as its ability to serve as a histone deacetylase (HDAC)

inhibitor, an energy metabolite to produce ATP and a G protein-coupled receptor (GPCR) activator (2). Intestinal bacteria use enzymes to split carbohydrates, with water, producing hydrogen, methane, carbon dioxide, acetate, propionate, butyrate, and lactate. The end products from bacterial fermentation engender energy for the colonic bacteria. A high fiber diet activates fermentation, which amplifies bacterial density and fecal mass, increasing the viscosity of the stool. Approximately, about 30 g of bacteria is generated for every 100 grams of carbohydrate made through fermentation (1). E. coli produces and secretes an enzyme, acetyl-CoA carboxylase, breaking down glucose into butyrate, acetate, and propionate (6). Four genes, accA, accB-accC, and accD, code for the translation of Acetyl-CoA carboxylase. These four genes for Acetyl-CoA carboxylase can modify the composition of fatty acids and monitor the rate of production. The four genes also monitor the over expression of ACCase with inserting one of the four subunits to activate antisense RNA expression (19) . Acetyl-CoA carboxylase hydrolyzes glucose into short chain fatty acids including:propionate, acetate, and butyrate. SCFAs are hydrophilic, soluble, and the bloodstream readily absorbs each SCFA. Many of the body's major organ systems as the nervous system, skeletal-muscle system, and tissues catabolize acetate directly. Propionate decreases the liver's production of cholesterol through the liver's ability to efficiently decay and clear Propionate, which blocks its synthesis. The SCFAs initiate apoptosis(1). Fermentation of glucose to produce SCFAs also constrain the development of disease-causing organisms by decreasing luminal and fecal pH (1). By lessening the pH, the expression of unfavorable bacterial enzymes decreases due to reduced peptide degradation and by the production of ammonia, amines, and phenolic compounds (1). In this study the gene, accA, was analyzed through qPCR quantification. The concentration of accA was measured after culturing E. Coli with high, medium, and low concentrations of glucose. The list of concentrations for glucose administered include: 15mM, 7.5mM, 5mM, 200 u M, 50 u M, and 0u M. For example, ECV304 human endothelial cells restricted the metabolism of glucose in response to high levels of glucose in the medium by decreasing the rate of glucose phosphorylation (8). The regulation of metabolizing glucose suggests that glucose phosphorylation is altered in vivo in response to high glucose levels (8). Also, the levels of quorum sensing was analyzed. Quorum sensing is communication between bacterial cells through a release of small signaling molecules called autoinducers. The autoinducers are released to regulate the aggregation of cells and genetic expression. The autoinducers of gram negative bacteria consists of homoserine lactones versus gram positive bacteria that are oligopeptides (35). We used qPCR to quantify the amount of gene copies for the accA gene. Absolute qPCR produced a standard curve for the genes from each bacterial grown in varied and set concentrations of glucose. Real-time PCR using the LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany) is accomplished by the ceaseless quantification of the PCR products. The method is rapid and easy for the quantitative recognition of microorganisms (17). The inhibition of genetic expression for accA will be inhibited through antisense RNA. Antisense RNA innately occurs in bacterial cells as an immune response to foreign genetic material, mainly foreign and viral DNA or RNA. The original protocell could constrain foreign and movable DNA through transcription of antisense RNA, complementary to its specific DNA target sequence (18). The asRNAs bind to the sequences flanking the ribosome-binding site and the start codon of the target mRNAs (15). They block ribosomes from detecting the RBS, and therefore inhibit translation (15). In the current study, antisense RNA was amplified through PCR of an antisense DNA sequence flanked with Xho1 and Nco1 restriction sites, designing primer sequences of 30bps each. The PCR product was ligated into the PHN1257 plasmid (Fig. 1). Competent bacterial cells were transformed with the recombinant IPTG-PT-asRNA plasmid called PHN1257. The total RNA was extracted for qPCR analysis to determine the number of gene copies for accA. Inhibiting genetic expression, at a intracellular level, was quantified after bacterial cells for E. Coli, were transformed with recombinant DNA, the PHN1257 plasmid, containing the DNA insert. The asRNA blocked the mRNA expression of accA. Finding the connection between the condition of the digestive system's microbiome, at a cellular and molecular level, and disease can lead to devising alternative means of treatments. We want to find how gene regulation with the different variations of growth factors as hormones, and the necessity for a high fiber diet may treat disease from the gut to the whole human body. Therefore, regulating the environmental, genetic, and the hormonal communication of signaling factors within the digestive tract can restore the homeostasis from the intestinal microbiome to other organ systems.

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METHODS

RNA Preparation Escherichia coli cells were grown on 25mL Luria Broth agar media plates. Transformed bacterial competent cells, with (+antisense)and (-antisense) PTasRNA expression vectors, were grown on LB agar plates with the kanamycin antibiotic and incubated at 37oC for 24hrs. Cells grown on agar plates were inoculated into 4mL of LB liquid media with (+) and (-) PTasRNA bacterial cells expression of asRNA for accA cultured with kanamycin. E.Z.N.A.® Bacterial RNA Kit allowed for rapid and reliable isolation of high-quality total cellular RNA from a wide variety of bacterial species. Up to 3mL of bacterial cell culture from the cells grown in the varied concentrations of glucose, the (+) asRNA, and the (-)asRNA cells were centrifuged at 4,000 x g for 10 minutes at 4oC. The medium was discarded and cells resuspended in 100uL Lysozyme/TE buffer. The solution was vortexed for 30 seconds. Incubaction occured at 30oC for 10 minutes in a shaker-incubator. The lysis buffer of 350uL with 25mg of glass beads were added. It was centrifuged for 5 minutes at maximum speed. RNA was extracted using HiBind® RNA mini columns through RNA wash buffers, I and II. The RNA was eluted with 50uL of DEPC water. Up to 1 x 109 bacterial cells were processed. The system combined the reversible nucleic acid-binding properties of Omega Bio-Tek's HiBind® matrix with the speed and versatility of spin column technology to yield approximately 50-100 u g RNA. cDNA Preparation We used the OneScript Reverse Transcriptase OneScript cDNA Syntheis kit by ABM. Approximately 1ug of RNA from each sample, High-glucose, Medium-glucose, Low-glucose, and (-)glucose was added to 0.5uM oligonucleotides(dT) (10uM). For the asRNA and (-) RNA, 1 ug of each total RNA extract was added to the initial primer/RNA and reaction mixture. The Initial Primer/RNA mix: 1ug of total RNA, 1uL of oligo(dt), 1uL of dNTP (10mM) mix, nuclease free-water for a 20uL reaction solution. The mixture was heated to 65oC for 5 minutes and then incubated on ice for 1 minute. The reaction mixture of 4uL 5X RT buffer, 0.5uL of RNaseOFF, and 1uL OneScript RTase was added to the initial primer/RNA mix in a 2uL microcentrifuge tube. The cDNA was synthesized by incubating the tube for 50 minutes at 42oC. The reaction was stopped at 85oC for 5 minutes and chilled on ice. The cDNA was stored at -20oC. PCR A Promega PCR Master Mix volume of 25uL was added to upstream and downstream primers each of 0.5uL specific for the accA gene target. The concentrations of two cDNA samples added to the PCR master mix were 190ng and 230ng plus nuclease free water. A 2X 1X upstream primer, 10u M 0.5–5.0u l 0.1–1.0u M, and a downstream primer, 10u M 0.5–5.0u l 0.1–1.0u M, DNA template 1–5u l ¡ 250ng Nuclease-Free Water was mixed into a 50u l PCR reaction mixture. The thermocycler was set to a Denaturation of a 2-minute initial denaturation step at 95 degrees celsius C. Other Subsequent denaturation steps were between 30 seconds and 1 minute. The Annealing step was optimized with annealing conditions by performing the reaction starting approximately 5 degrees Celsius below the calculated melting temperature of the primers and increasing the temperature in increments of 1 degree Celsius to the annealing temperature. The annealing step was set at 30 seconds to 1 minute in 52 degrees Celsius. For the extension reaction with Taq polymerase, we allowed 1 minute for DNA to be amplified at 72 degrees Celsius with a final extension of 5 minutes at 72–74 degrees Celsius. The PCR thermocycler completed 40 cycles of amplification. Plasmid Assembly The condition of the PCR products, without primer dimers and contamination, for the target gene accA, were detected through agarose gel electrophoresis. The PCR products were confirmed without primer dimers and contamination, the PCR products and the PTasRNA expression vector of the plasmid PHN1257 were digested with the restriction enzymes XhoI (upstream) and NcoI (downstream) (New England Biolabs XhoI- catalog R0146S- 1,000 units and NcoI- catalog R0193S- 1,000 units). Each microcentrifuge tube was placed in the incubator for 30 minutes at 37oC. A heat block was heated to 90oC. The PCR products of the accA gene were ligated into the PHN1257 plasmid by mixing 1uL of the DNA insert with 2uL of the plasmids, adding 5uL of ligation mix, and then placing the tubes into a heat block of 90oC for 15 minutes. (Takara Ligation Kit 6023). Competent bacterial cells were transformed with the PHN1257 plasmid plus the antisense DNA insert of accA, for expressing (+)asRNA and (-)asRNA. The competent cells were incubated with the recomibinant DNA for 45 minutes at 37oC. Real time PCR The cDNA from H-glucose, M-Glucose, L-glucose, and (-) was diluted into a 1:8 serial dilution. We pipetted 8uL of diluted cDNA into all wells of a clear 96 well plate. A 0.4ul of each primer pair mixture was pipetted into a microcentrifuge tube with 10uL of SensiMix SYBR MasterMix, and with 1.2uL of DEPC water. The SYBR reaction mixture of 12uL was then pipetted into the wells with the diluted and undiluted cDNA. Optical film sealed the plate. To suspend all liquids to the bottom of the wells, we Centrifuged the PCR plate for 2 minutes at 2500rpm. The RT-PCR Amplification Protocol (Roche Lightcycler 480 machine) began with a 3 min step at 95°C for 40 cycles with a step of 10 sec at 95°C, 45 sec at 65°C,

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and 20 sec at 78°C with One step of 1 min at 95°C. One step of 1 min at 55°C 80 cycles of 10 sec each starting at 55°C with a 0.5°C increment at each step up to 95°C. For the Roche Lightcycler 480 machine the 96-well clear Roche plates were used for SYBR green detection.

RESULTS

The RNA Concentration and accA Genetic Expression of each Glucose Sample Escherichia coli MG1655 bacteria was grown in LB broth overnight at 37 o C with 15mM, 7.5mM, 5mM, and 0 mM of glucose concentration for high-glucose, medium-glucose, low and zero glucose as a control, respectively (Fig. 2). RNA was extracted from each sample and the concentrations determined by the Implen NanoPhotometer 250. The RNA concentrations for each sample measured were 1392ng/uL for high-glucose, 797 ng/uL for medium-glucose, 608 ng/u L for low-glucose, and 179 ng/uL for the control. The high-glucose sample had the highest amount of RNA compared to the medium to low and to the control. The RNA for each sample was reverse transcribed into first strand cDNA and absolute quantification with qPCR was used to measure the amount of the target gene, accA, produced by each sample. High-glucose had a Cp of 12.28 and the concentration of accA was 4.21E3 ng/u L. The Cp of sample medium-glucose equaled 16.51 with a concentration of 3.75E2 and the low-glucose Cp was 14.08 with target gene concentration of 1.50E3 (Fig. 3). The control group had a Cp of 17.64 with a target gene concentration of 1.96E2. After loading a 96-well plate for qPCR with 5-fold dilution standards of each sample, the standards of each sample were used to calculate a standard curve (Fig. 2) For the glucose concentrations of 200 uM, 50 uM, and 0uM compared to their RNA concentrations, the standard deviation was 148 SD 204, 190 SD 252, and 107 SD 76, respectively. Comparing 200 uM to its control group, the difference between the samples was statistically significant. The cells grown in 200uM had a more significant and larger production of accA, with a p-value of 0.038, than cells grown in 0uM of glucose.

The qPCR results of asRNA for the target Gene accA Recombinant DNA was produced when the PCR product of the gene insert, accA was ligated into the plasmid PHN1257 (Fig. 1), that was engineered to amplify antisense RNA. The PCR product and the IPTG-PT-asRNas plasmid of PHN1257 were cut with the restriction enzymes XhoI and NcoI. The primers (Table. 1) each contained an extra 3 to 4 bps of nucleic acids to accompany the sequences for Xhol and Nco1. The primers flanked the target DNA, which totalled to 150 base pairs. The antisense sequence was constructed in a specific orientation where the restriction enzymes, which NcoI normally flanks the forward primer, reversed positions. The XhoI was repositioned to flank the forward primer, and the Nco1 would flank the reverse primer, creating the antisense sequence when inserted into the plasmid for PHN1257. The total RNA concentration for the untransformed bacterial cells, or the positive control, equaled 739.44 ng/u L with an A260/A280 of 1.9. The positive control, No-asRNA, had a miRNA concentration of 334.98 ng/u L, including an A260/A280 of 2 (Fig. 3). The RNA concentration for transformed bacterial cells with the asRNA of accA PHN1257 plasmid was 279.28ng/u L, having an A260/A280 of 2.011. The miRNA concentration of the cells with asRNA measured to 240.90 ng/u L and an A260/280 absorbency of 2.073 (Fig. 3). The gene of accA was successfully suppressed by asRNA in vitro with 63 ng/u L measured for bacteria cells transformed with the recombinant antisense PHN1257 plasmid DNA. The bacterial cells with the PHN1257 plasmid but without the antisense gene target and insert produced 421.69 ng/uL for accA. There was a 138asRNA for accA. A p-value of 0.027 showed highly significant data for the accA gene target concentration of PHN1257(+)asRNA versus PHN1257(-)asRNA, or without asRNA. The gene of accA was successfully suppressed by asRNA in vitro with 63 ng/uL measured for bacteria cells transformed with the recombinant antisense PHN1257 plasmid DNA. The bacterial cells with the PHN1257 plasmid but without the antisense gene target and insert produced 421.69 ng/uL for accA (Fig. 4). There was a 138versus cells transcribing the asRNA for accA. A p-value of 0.027 showed highly significant data for the accA gene target concentration of PHN1257(+)asRNA versus PHN1257(-)asRNA, or without asRNA. Quantification of Lux-S Gene Expression Bacterial cells were transformed with the PHN1257 plasmid expressing asRNA to inhibit the genetic expression of the accA gene. RNA was extracted from cells grown with 25u M glucose and 5u M of glucose. The cells grown with glucose in the medium were also transformed with antisense expressing IPTG-PT-asRNA inducible PHN1257 plasmids.Cells were cultured without (-)glucose but the expression of accA was also inhibited. A control, without (-)glucose and (-)asRNA in vitro translation, was compared to each sample. The number of gene copies for Lux-S was measured through qPCR and absolute quantification with a p-value¡0.05,(Table. 2). The (-)glucose-(+)asRNA sample had a gene copy number of 199 and 2511 Lux-S copies for (-)glucose-(-

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)asRNA. For the 25u M glucose-(+)asRNA and 5u M glucose-(+)asRNA produced 39,810 and 1x10 6 gene copies of Lux-S, respectively (Fig. 5). The samples with 5u M glucose-(+)asRNA exhibited the highest amount of expression for Lux-S, which displays an increased amount of quorum sensing releasing more autoinducer-2 molecules, directly affected by an increase sensitivity to glucose.

DISCUSSION

We assembled recombinant DNA plasmids from the IPTG-PT-asRNA of the PHN1257. The constructed plasmid of PHN257 consisted of flanking inverted repeats that flank for the target DNA, rebuilding a paired double-stranded RNA termini that inhibited the transcription of accA (15). The extracellular processes of the microbiome were measured through increasing the gradient of glucose concentration. Transcription of intracellular molecules were determined through qPCR. High levels of glucose increased the expression of accA. Inhibition of accA was confirmed through qPCR results of 63 ng/u L for accA(+)asRNA versus 422 ng/u L for accA(-)asRNA. The amount of intercellular quorum sensing between bacterial cells was quantified through qPCR. The volume of Lux-S wasd determined through qPCR. The qPCR results demonstrated that the end yield of Lux-S and autoinducers, AI-2, cells are dependent on the supply of glucose. The four genes accA-accD code for the subunits of the complex, Acetyl-CoA Carboxylase, which catabolizes dietary fiber in the form of glucose to begin many biosynthetic processes. Acetyl-CoA carboxylase (ACC) initiates the first step of fatty acid synthesis. During fatty acid synthesis, malonyl-CoA is formed from acetyl-CoA, using energy from ATP and bicarbonate production (22). Glucose is hydrolyzed into pyruvate, which is made into acetyl-CoA, forming acetate. Through the Wood-Ljugdahl pathway, pyruvate loses two hydrogens, carbon dioxide loses an oxygen, altered into carbon monoxide, and a methyl group is added to this reduction to make acetyl-CoA into Acetate (20). Butyrate is produced when two molecules of acetyl-CoA combine into acetoacetyl-CoA. The acetoacetyl-CoA is transformed into butyryl-CoA, or butyrate (20). Propionate is composed through the succinate or acrylate pathway (41). PEP is broken into pyruvate which is further metabolized with water into succinate. The succinate is reduced into propionyl-CoA, forming propionate. Through the acrylate pathway, lactate loses an oxygen, forming propionate (20). Therefore, SCFAs are highly significant for initiating the downward cascades for hormonal responses, regulating metabolism, controlling hunger signals to the brain, and affecting many psychological behaviors. However, the rate of forming SCFAs is dependent on the glucose concentration within the lumen of the intestines (16). Ferrais et al., measured the luminal glucose osmolarity and concentrations of the small intestines. They found the assumptions of luminal glucose concentrations being 50mM to 500mM to exhibit some errors (16). The previous studies measured glucose concentrations but did not recognize how osmolality is affected by Na+ and K+ salts, amino acids, and peptides (16). Ferrais et al., discovered the average of SI luminal osmolalities were approximately 100 mosmol/kg, which were mainly hypertonic results. For an animal's diet, the SI glucose concentrations averaged 0.4-24 mM and ranged with time over a large amount of a SI region from 0.2 to 48 mM. E. Vinalis et al found that high concentrations of glucose lessened the absorption of glucose in endothelial cells called ECV304 cell lines. There was a 60The high glucose osmolities resulted in reduced Vmax values for 2-deoxyglucose uptake with a constant Km, calculated from the Michaelis-Menten kinetic enzyme equation (16). However, from our results, we found the genetic expression of accA to be proportional to increasing glucose concentrations in E.Coli bacterial samples. After we cultured E. coli with different levels of glucose, the RNA concentrations for each sample measured were 627 ng/u L for high-glucose, 452.88 ng/u L for medium-glucose, 361.72 ng/u L for low-glucose, and 137.60 ng/u L for the control. The concentration of accA, in ng/u L, for each sample included: 4210, 375, 150, and 196 for H-glucose, M-glucose, L-glucose, and No-Glucose respectively. We reached these specific results for genetic expression amplified in conjunction with increased glucose concentration because the four subunits for the acetyl carboxylase complex seem to be regulated only by the accBC lac operon and each gene, accA and accD, are entirely independent of the accBC lac operon. When accB is overexpressed, accBC transcription is blocked, but the overexpression of the other three gene products don't affect the accBC operon transcript levels (22). When there is a high glucose concentration, the availability of accB increases, and the transcription of the accBC lac operon is greatly reduced even after an exposure to a small amount of accB. Although a high glucose concentration may have stymied the production of accBC, it did not seem to inhibit the amplification of accA, our target gene and DNA sequence of study. E. Coli cells were grown in 25u M and 5u M of glucose and transformed with antisense PHN1257 expressing IPTG-PT-asRNA plasmids. Cell samples with 5u M glucose-(+)asRNA produced the highest amount of Lux-S gene (28).

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Lux-S is needed to produce autoinducer two. Lux-s monitors the amount of biofilm, flagellum movement, and monitors virulence. Biofilm increases the movement of bacteria into the bloodstream (35). The 5u M glucose-(+)asRNA sample showed the most expression of Lux-S because Wang et al., (2005) found adding 0.8glucose to their bacterial culture and growth medium increased the activity at the promoter site of the Lux-S gene. A glucose concentration of 5u M results in a normal exogenous osmolarity surrounding bacterial cells within the microbiome of the lower digestive tract (28). Because cells were overly saturated with a 25 u M concentration of glucose, we expected to find more release of autoinducer-2 signaling molecules with more regulation of genetic expression. However, the over saturation of glucose at 25 u M did not result in more genetic expression of Lux-S. Jesudhasan et al. (2010) proved that higher levels of autoinducer-2 did not lead to increased genetic expression in Salmonella Typhimurium when cultured in high concentrations of glucose (29). By transforming E.coli bacterial cells with IPTG-PT-asRNA inducible vectors, the Lux-S gene was inhibited. When the Lux-S gene is mutated or suppressed, it becomes more responsive to the concentration of glucose. Therefore, more transcription of Lux-S was observed in cells cultured with 5u M glucose and less in activity from the 25u M sample. Because glucose delays the movement of the Lux-S mutant strain, but does not inhibit bacterial growth, each (+)asRNA sample appeared smaller and defected. Osaki et al., (2006) showed that Lux-S mutants can be smaller in diameter with 8.0 mm versus 12.3 mm in its wild type (30). Figure. shows bacterial colonies expressing antisense RNA with a smaller size versus cells without antisense RNA. Cell samples with (+)asRNA transcription were smaller in size (30). Bacterial samples transformed with IPTG-PT-asRNA inducible PHN1257 plasmids, designed to inhibit the accA gene, had the least measure of Lux-S expression. The (-)glucose-(+)asRNA sample had the lowest amount of Lux-S expression of 199 gene copies. As a result, when the Lux-S gene is mutated with less expression, the biofilm is thinner, looser, and displaying an appearance of bacterial cells with defects. Lux-S forms a thicker and more viscous biofilm, which results from a large amount of DNA released. The ample amounts of DNA released maintains a more solid macromolecular matrix and consistency of the biofilm (39). Lux-S is required for AI-2 production and for regulating gene expression in the early-log-growth phase. Pathogenic bacteria depend on biofilm formation to attach to epithelial cells and tissues, spreading infectious diseases. Pathogenic bacteria infects host cells by accumulating AI-2, exogenously, which then increases the amount and consistency of the formation of biofilm (37). Streptococcus, a gram negative bacteria, results in the death of 2 million people each year. Streptococcus lives symbiotically with other microflora in the nasopharynx region of the respiratory system. After a month of commensal habitation, Streptococcus begins to infect other parts of the body, leading to disease. To form biofilm for infection, the Lux-S gene must be activated. Its excessive amplification and expression leads to a denser texture of biofilm (39). Lux-S monitors virulence through regulation of the process of generating biofilm in the nasopharynx in mice with pneumonia. Flagella expression increases with Lux-S mutation in the lungs and bloodstream. The Lux-S mutants can infect the lungs or the bloodstream more rapidly than its wild-type strain (36). Flagellin increases inflammation by activating TLR5 pathways, which translates pro-inflammatory genes within the MAPK pathways. Flagella modulates virulence and pathogenesis by allowing a more rapid motility of bacteria, infecting the colonization of host cells, and assisting infectious bacterial cells with entering the mucosal layers (41). Pathogenic bacteria need to determine the autoinducer signals specific to their particular species. Pathogenic bacteria require a specific order and assortment of virulent genes to infect, spreading disease. For example, the different residues or R-groups of amino acids within autoinducers bind to LUXR protein receptors in a conformation specific to the amino acid side chains within the binding sites. The orientation of the AI to protein receptor binding produces varied types of side chain lengths and amino acid substitutions (40). Microbiota in the gut respond to Lux-S transcription differently than in the respiratory system. For example, pathogenic bacteria as E. coli EPEC has intraspecie signals that help to colonize the small intestines. The small intestines is void of many commensal microflora. The EHEC pathogenic form of E. coli conducts signals with other bacterial cell types and with host cells. EHEC communicates through quorum sensing with other normal large intestine microflora. EHEC infects through activating the genes called Locus of Enterocyte Effacement or LEE. The genes of LEE are required for A/E lesions to form when EHEC cells attach to and efface from epithelial cells, amplifying the level of pathogenicity (34). Autoinducer 2 requires the genetic expression and translation of Lux-S. When Lux-S and autoinducer-2 is less regulated and overexpressed, infectious bacteria can propagate in stressful environments with high acidity and salinity (32). Kendall et al., (2008) demonstrated S.pyogenes adapting to acidic conditions when the luxS/AI-2 system was unregulated. Autoinducer 3 is not dependent on

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expression of the Lux-S gene. Lux-S genetic mutants impedes the production of AI-3 (31-32). As a result, the pathogenic E.coli, EHEC, collects signals from host cells in the form of hormones as epinephrine. AI-3 has the ability to communicate with the transmembrane protein called Qsec (31). Bone marrow white blood cells, as macrophages, can be incubated with synthetic 3-oxo-C12-HSL and C4-HSL, for 24 hours. The induced apoptotic activity of 3-oxo-C12-HSL can be demonstrated in neutrophils and monocytic cell lines U-937 and P388D1, respectively (7). Cells treated with 3-oxo-C12-HSL can reveal morphological alterations indicative of apoptosis (3). Qualitative data of apoptosis can be assayed with BIORAD with live cell fluorescence microscopy. Bone marrow cells can be cultured with an assay called the pSIVA REAL-TIME Apoptosis Fluorescent Microscopy Kit. The assay of apoptosis can give us real time data-analysis of apoptotic signaling at the cell surface (17).

CONCLUSION

We attempted to learn more about the fluctuations within the exogenous composition of the extracellular matrix and from the effects of genetic mutations on the intracellular downward biochemical cascades. We intended to show an intense and acute budding of pathogenesis when the intracellular processing of intercellular signaling molecules are combined with genetic and external changes that innumerably alter the intestinal microflora.The results observed displayed the RNA concentration for bacterial cells, expressing asRNA of accA, included a concentration of 279.28 ng/u L, having an A260/A280 of 2.011. The miRNA concentration of the cells with asRNA measured to 240.90 ng/u L and an A260/280 absorbency of 2.073, with a p-value¡0.05. The concentration for cells without antisense RNA was 422 ng/u L compared to 63ng/u L for cells expressing asRNA. Antisense RNA transcription also occurs in bacteria naturally. Naturally occurring asRNAs were first observed in bacteria more than 30 years ago and were approximated even earlier for the bacteriophage of lambda (22). In archaea, the first case of the antisense control of gene expression was reported in 1993 for the extremely halophilic prokaryotic cells called the Halobacterium salinarum, with an asRNA complementary to the first 151 nucleotides (nt) of the transcript T1 (22). From dietary fiber, or undigested carbohydrates, SCFAS are produced and can catalyze hormonal signals within enteroendocrine cells. SCFAS can bind to G-protein coupled receptors, leading to a downward cascade of reactions within endocrine cells. For example, SCFAs bind the G-protein coupled receptor 41, which then causes the secretion of the hormone PYY. Peptide YY increases the rate of digestion and conserves the energy from the diet (21). Including more dietary fiber in the Western Style diet is one of the best ways to restore balance and a healthy condition of the intestinal microbiome. However, there may be more alternatives for treating dysbiosis within the microbiome of the gut. Since 70improve insulin sensitivity, fight obesity, eradicate colon cancer cells, and reduce mental disorders as depression, the human gut microbiota may be a source of a conduit for treating many human diseases. For example, there has been found a tumor-inhibiting molecule released by a probiotic strain of bacteria. The L. casei strain of ATCC 334 produces ferrichrome, which delays the metastasis of colon cancer by activating the c-Jun N-terminal kinase pathway of apoptosis (27). Increasing production of ClpB, a chaperone protein secreted by E.coli, reduced food intake, limit meal patterns, and stimulated intestinal hormones in mice as a glucagon-like peptide 1, an antihyperglycemic protein (27). Another study found therapeutic opportunities for inflammatory diseases through isolating 17 clostridium strains and re-engineering mixtures of those strains, which decreased colitis and increased Treg cells in rodents. Inflammation of the airways due to allergies and inflammatory colitis have been shown to be remedied by interleukin-10, a cytokine expressed by the bacteria, Lactococcus lactis (27). Isolating microbial organisms with the possibility of providing alternative therapies for disease is extremely favorable for future research. Synbiotics is a territory minutely explored, but is gaining noticeability. For future studies, we are greatly interested in the live cell imaging of bone marrow cells lines cultured with 3-oxo-C12-HSL, displaying real time apoptotic activity at the cell membrane level. The 3-oxo-C12-HSL is a P. aeruginosa signal molecule homoserine lactone reported to inhibit function of PPARs in mammalian cells, combating lung disease (24). Bacterial cells cultured in high-glucose to a zero glucose concentration showed a decreasing range of genetic expression for our target gene, accA. Like we hypothesized an increased amount of available glucose showed the highest measure of transcription of accA. We silenced the accA gene with antisense RNA, using a, IPTG-inducible vector called PHN1257. There was a 138antisense PHN1257 versus cells with no antisense RNA in vitro transcription. We wanted to model how high levels of glucose, from high fiber intake, increases production of accA into mRNA for translation into acetyl-CoA carboxylase enzyme, which produce SCFAs. We also attempted to show how a gene knockout of accA with asRNA

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can decrease genetic expression. In many diseases of the colon, an excess of glucose or hyperglycemia can lead to inhibition of genetic expression of pertinent genes needed for metabolism and fatty acid synthesis. We found that SCFAs, metabolites from the fermentation of insoluble fiber and glucose by E.Coli bacteria, has many beneficial properties and characteristics. Butyrate can bind transmembrane proteins, leading to a cascade of cellular reactions as apoptosis through quorum sensing. The intracellular signals induce communication between bacterial cells to endothelial cells of the large intestines. Therefore, with the inhibition of the gene accA, the fermentation of glucose can be delayed leading to decreased formation of the catabolite of butyrate and silencing the quorum sensing between cells. The microbiome of bacterial cells can communicate with the gut, using a variety of chemical idioms, which, their host cells can detect (25). This review has considered the relevance for the link between an individual's overall health to bacterial cells' sensitivity to glucose, and the availability of metabolites. However, we focused on presenting evidence for the impact of effects from bacterial cells potential for detecting host signalling molecules upon (9). A group of chemical molecules for bacterial language expression and transfer includes homoserine lactones, which are the most heavily studied. When infection occurs, homoserine lactones are formed, and interrelate with the immune system (26). Hormones affect the balance of flora within the microbiome. According to Hur Vi. Linn et al (2015), SCFAs are also subunits of signaling molecules. The G protein-coupled receptors called Free fatty acid receptor 2 (FFAR2, GPR43) and FFAR3 (GPR41) have been identified as receptors for SCFAs. Acetate stimulates FFAR2 in vitro; propionate presents similar traits of binding to a receptor and triggering downward cascade responses on FFAR2 and FFAR3; and butyrate only activates FFAR3 (26). When bacterial genetic expression of the genes accA, accBC, and accD are inhibited, the production of SCFAs is disrupted, delaying hormonal responses. The fermentation of glucose by E.Coli leads to SCFAs binding to G-coupled protein receptors that increase or decrease cell-to-cell signaling (12). The SCFA receptors FFAR2 and FFAR3 are both expressed in the intestine and maintain symbiosis with adjacent enteroendocrine cells in the mucosal lining of the digestive tract that express the Peptide YY (PYY). FFAR3 deficiency in mice was associated with a reduced involvement of bacteria from the microflora, which allowed an increased expression of PYY in the plasma (12). The purpose for our study was to construct a culmulative understanding of the processes within the inner mechanisms of the intestinal microbiome. The microbiome is a vast organ, so our aim was to attempt to compile a small part of its large navigational map to identify possible locales for future synbiotic research and study.

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Table 1(on next page)

Primers for amplifying the Lux-S gene, antisense RNA for accA, and the accA gene.

Table 1. Primers for PCR Amplification and qPCR Analysis. To produce antisense RNA for accA inhibition the restriction enzyme site for XhoI was sequenced with the forward primer for accA. The restriction enzyme NcoI was added to the reverse primer for accA. Through PCR amplification, the pcr products were composed of antisense DNA for the accA gene. The PCR product would be inverted when inserted into the IPTG-PT-asRNA inducible PHN1257 plasmid, forming recombinant DNA plasmids for asRNA transcription in vitro. The plasmids with recombinant antisense DNA were transformed into bacterial competent cells for qPCR analysis of the accA gene. The primers for the Lux-S gene were applied to quantify the number of gene copies from qPCR analysis for cells grown in glucose enhanced samples. The primers for the accA gene were used for the quantification of the accA gene from the samples cultured with an increasing gradient of glucose solutions.

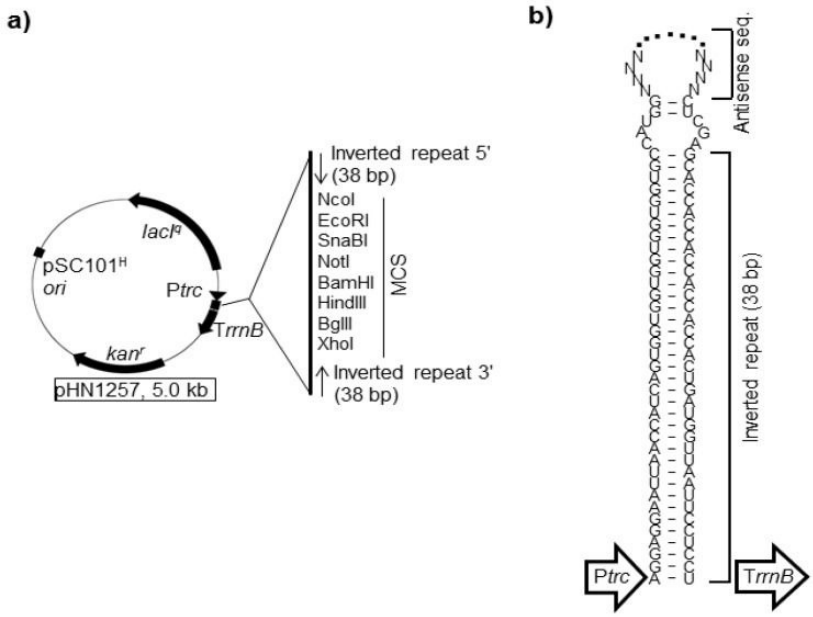
Primers	DNA Sequence
XhoI+accA Forward	GAGATGAGTCTGAATTTCCTTGATT
NcoI+accA Reverse	TGGCAGTTCATCGCTTTTTTCAC
Lux-S Forward	CATACCCTGGAACATCTGTTTGC
Lux-S Reverse	AGTTCCTGCACTTTCAGCACATC
accA Forward	TCATCACCTTTATCGACACCCC
accA Reverse	TTCACTTTATCGCCCACGCC

Figure 1(on next page)

Displays an image of the antisense RNA Vector, PHN1257.

Fig. 1 Plasmid Assembly and Gene Silencing the PCR products and the PTasRNA expression vector of the plasmid PHN1257 were digested with the restriction enzymes XhoI (upstream) and NcoI (downstream). After the restriction digestion, the antisense PCR product was ligated into the IPTG-PTasRNA inducible vector of PHN1257 at the multiple cloning site.

doi: 10.3390/ijms15022773

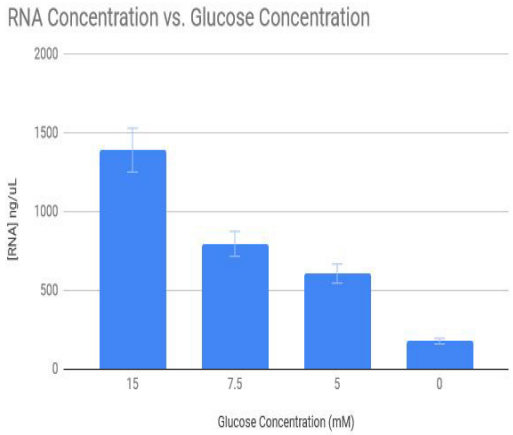


https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3958881/figure/f6-ijms-15-02773/

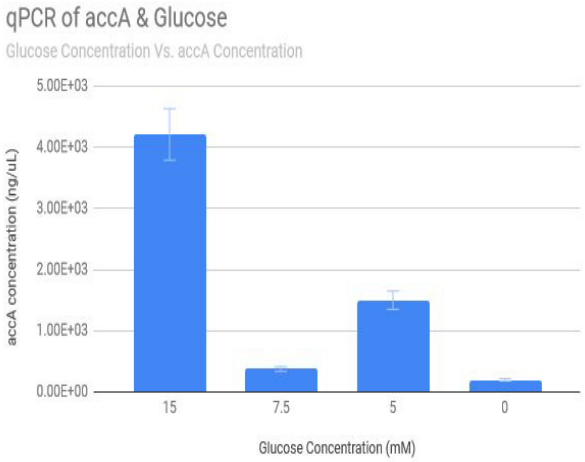
Figure 2(on next page)

Displays results for glucose affecting dsDNA and RNA concentration.

A)



B)



C)

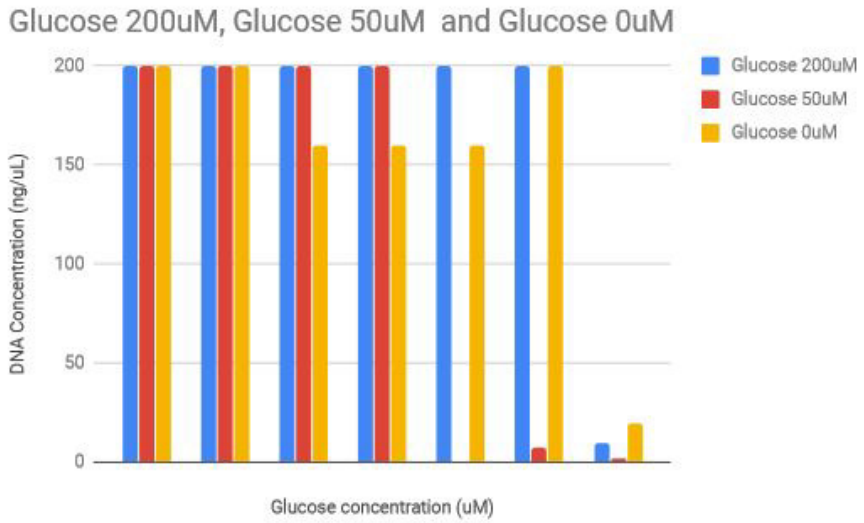


Fig. 2 A) Glucose concentrations of 15mM, 7.5mM, 5mM, and the control samples each had RNA concentrations, in ng/uL, 1392, 797, 608, 179, respectively. The bacterial sample with 15mM glucose, a high concentration, had the largest measure of RNA, noting a direct proportional link between glucose and genetic expression in gram negative bacteria as E.coli. **B)** Includes qPCR results for 15mM, 7.5mM, 5mM, and 0mM. The samples with 15mM and 5mM glucose displayed the most genetic activity of accA transcription, measuring accA concentrations at 4,210 ng/uL and 1,500ng/uL respectively. The accA qPCR concentrations for 7.5mM equaled 372 ng/uL and 196 ng/uL for 0mM. **C)** Displays OD260 results for samples of E.coli grown in medium enhanced with 200uM, 50uM, 0mM of glucose.

Figure 3(on next page)

The qPCR results for quantifying accA concentration and the gene copy number.

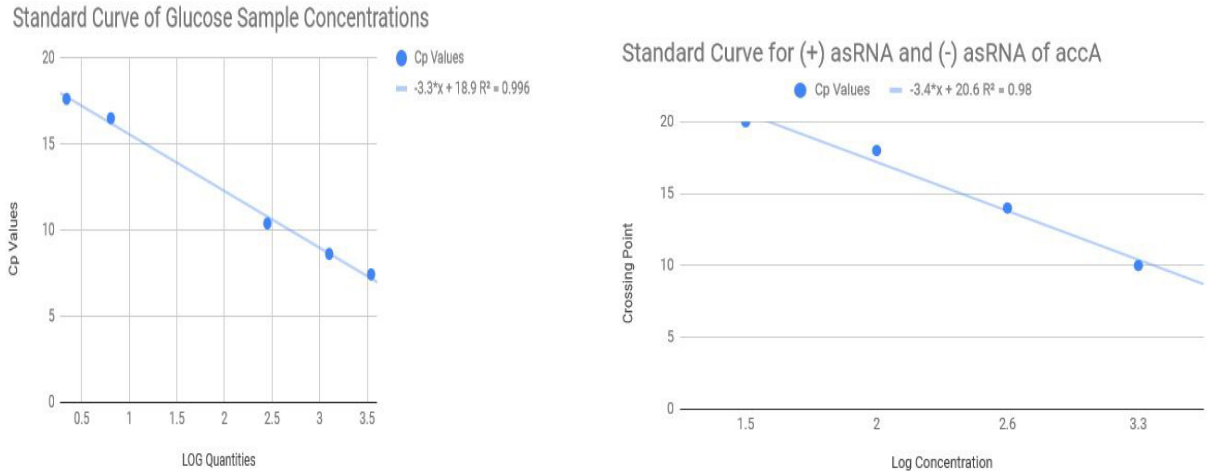


Fig. 3 Standard Curves for Quantifying Gene Copies The RNA for each sample was reverse transcribed into first strand cDNA and absolute quantification with qPCR was used to measure the amount of the target gene, accA, produced by each sample. High-glucose had a Cp of 12.28 and the concentration of accA was 4.21E3 ng/uL. The Cp of sample medium-glucose equaled 16.51 with a concentration of 3.75E2 and the low-glucose Cp was 14.08 with target gene concentration of 1.50E3. The gene of accA was successfully suppressed by asRNA in vitro with 63 ng/uL measured for bacteria cells transformed with the recombinant antisense PHN1257 plasmid DNA. The bacterial cells with the PHN1257 plasmid but without the antisense gene target and insert produced 421.69 ng/uL for accA. There was a 138% percent difference between cells not expressing asRNA versus cells transcribing the asRNA for accA. A p-value of 0.027 showed highly significant data for the accA gene target concentration of PHN1257(+)asRNA versus PHN1257(-)asRNA, or without asRNA.

Figure 4(on next page)

Gene Copies for Lux-S determined through qPCR measurement .

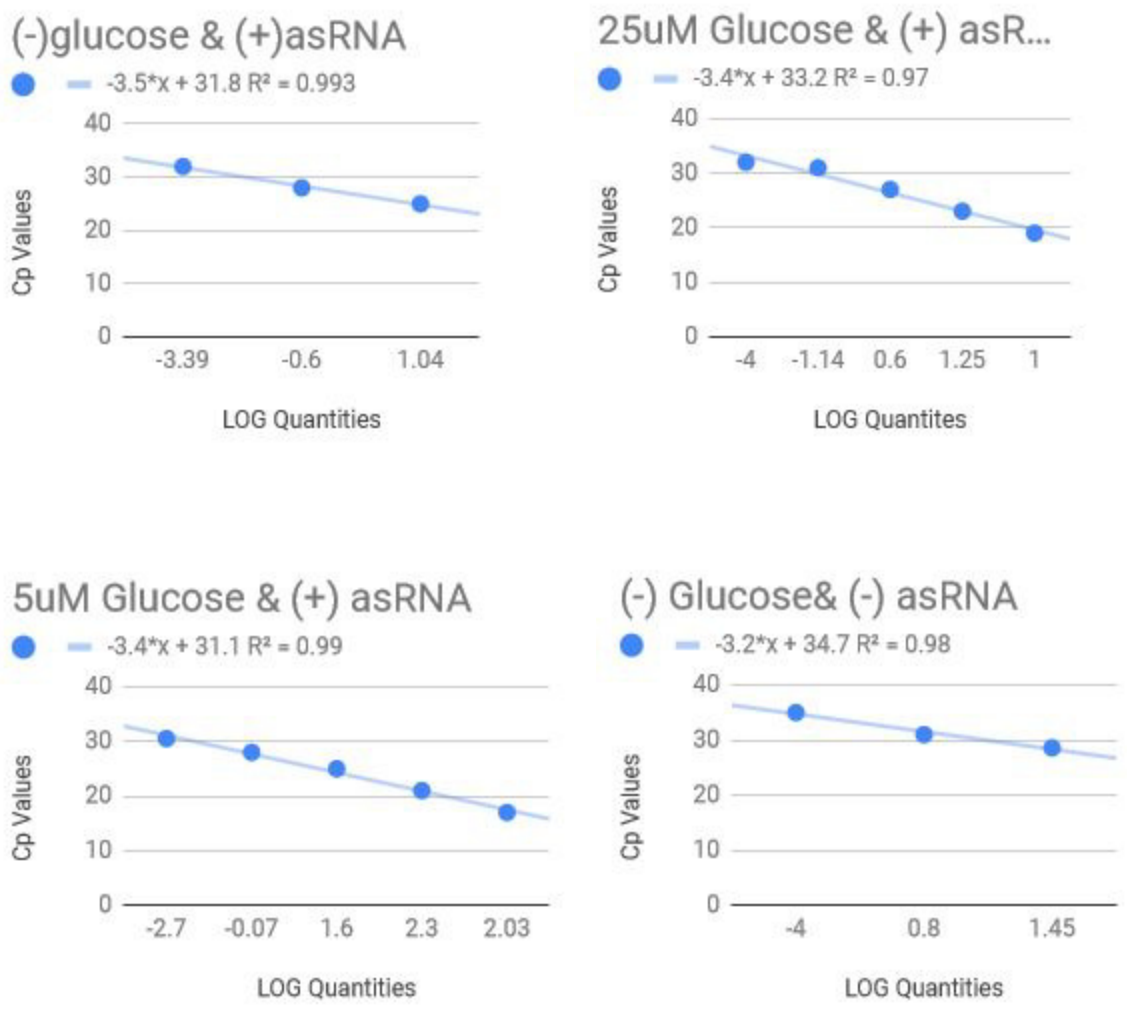


Fig. 4 qPCR Results for Lux-S The number of gene copies for Lux-S was measured through qPCR and absolute quantification. The (-)glucose-(+)asRNA sample had a gene copy number of 199 and 2511 Lux-S copies for (-)glucose-(-)asRNA. For the 25uM glucose-(+)asRNA and 5uM glucose-(+)asRNA produced 39,810 and $1x10^6$ gene copies of Lux-S, respectively.

Table 2(on next page)

Anova Results of each experimental sample for quantifying qPCR gene copies.

ANOVA Results of qPCR Gene Copies

	Lux-S-glu/(+) asRNA	Lux-S-glu/(+) asRNA	(-)glu(-)asRNA	accA (+)glu/accA (-) glu	accA (+)glu/accA (-) glu
1	2511	316228	1000000	186	100
2	2000	26915.4	1000000	79	32
3	745	17783	39810	18	5
4	199	251	151286	7.4	3
n	4	4	4	4	4
X	1363.750	90294.350	547774.000	72.600	35.000
s	1074.010	151028.260	524165.009	81.920	45.306
X_{ave}	127907.940				

total	19	1798315304447.048

source	df	SS	MS	F	P-value
treatments	4	905636341810.108	226409085452.527	3.8044	0.0250
error	15	892678962636.940	59511930842.463		
total	19	1798315304447.048			

Figure 5(on next page)

Graph of each sample with LuxS, expressing asRNA, enhanced with glucose, without glucose, and without asRNA.