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The effect of quercetin on genetic expression of the commensal gut microbes *Bifidobacterium catenulatum, Enterococcus caccae* and *Ruminococcus gauvreauii*

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ABSTRACT

Quercetin is one of the most abundant polyphenols found in fruits and vegetables. The ability of the gut microbiota to metabolize quercetin has been previously documented; however, the effect that quercetin may have on commensal gut microbes remains unclear. In the present study, the effects of quercetin on the commensal gut microbes *Ruminococcus gauvreauii*, *Bifidobacterium catenulatum and Enterococcus caccae* were determined through evaluation of growth patterns and cell morphology, and analysis of genetic expression profiles between quercetin treated and non-treated groups using Single Molecule RNA sequencing via Helicos technology. Results of this study revealed that phenotypically, quercetin did not prevent growth of *Ruminococcus gauvreauii*, mildly suppressed growth of *Bifidobacterium catenulatum,* and moderately inhibited growth of *Enterococcus caccae.* Genetic analysis revealed that in response to quercetin, *Ruminococcus gauvreauii* down regulated genes responsible for protein folding, purine synthesis and metabolism. *Bifidobacterium catenulatum* increased expression of the ABC transport pathway and decreased metabolic pathways and cell wall synthesis. *Enterococcus caccae* upregulated genes responsible for energy production and metabolism, and downregulated pathways of stress response, translation and sugar transport. For the first time, the effect of quercetin on the growth and genetic expression of three different commensal gut bacteria was documented. The data provides insight into the interactions between genetic regulation and growth. This is also a unique demonstration of how RNA single molecule sequencing can be used to study the gut microbiota.

1. Introduction

Quercetin is one of the most abundant polyphenols found in fruits and vegetables with an estimated daily intake ranging from 0 to 30 mg/day [1–5]. The highest concentration of quercetin is found in foods such as onions, berries, kale, and apples [1,2]. It is known to be a potent anti-oxidant, a free radical scavenger of both reactive oxygen and reactive nitrogen species, and binds with transitional metal ions [2,6]. Over the last few decades, interest in the functional properties of quercetin has increased as research continues to demonstrate its ability to inhibit diseases such as cardio-vascular disease, inflammation, cancer, diabetes, and obesity [1,2,6].

Although a number of studies have provided evidence that quercetin is beneficial to human health, it unfortunately has low oral bioavailability [7,8]. Quercetin is usually ingested in the form of a β-glycoside, conjugated to a sugar moiety [2,9]. Glucose conjugated quercetin can be absorbed in the small intestine; however, other conjugates cannot be absorbed and will be passed into the colon [8,10]. It has been proposed that 90–95% of ingested polyphenols are not absorbed, and therefore enter the large intestine

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where they encounter the resident gut microbiota [7,8]. The presence of polyphenols in the colon, and their subsequent interaction with the gut microbiota, may partially explain why eating a diet high in fruits and vegetables reduces risk of gastro intestinal cancers and other age related diseases [11,12].

The ability of the gut microbiota to chemically modify polyphenols is well known. In fact, most metabolites of ingested polyphenols isolated from human blood and urine samples are designated byproducts of colon metabolism [13]. For quercetin in particular, studies have pre-established that non-absorbed quercetin is transformed by the gut microbiota into phenolic acids [6,8,12,14]. Research has found that the bacterial strains *E. gilvus*, *S. lutetiensis*, *E. coli*, *L. acidophilus,* and *W. confusa* are able to degrade quercetin to varying degrees [15] and that *C. perfringens* and *B. fragilis* produce 3,4-dihydroxyphenylacetic acid from quercetin [16,17]. These studies also demonstrate that the reported modulations reduce quercetin's anti-oxidant properties [16,17]. Therefore, it is well documented that the gut microbiota is able to affect the chemical properties of quercetin [15]. However, the effect of quercetin on the phenotype and genetic expression of the resident gut bacteria is not as well characterized.

Until now, research studying the effects of quercetin on gut microbes has been principally based on evaluating changes in cell growth. Many of these studies use whole compounds that contain multiple polyphenols, including quercetin, and involve the use of pathogenic bacteria [17,18]. In one previous study, the effect of quercetin on the growth of *Bifidobacterium catenulatum (B. catenulatum), Enterococcus caccae (E. caccae),* and *Ruminococcus gauvreauii (R. gauvreauii)* was tested, and the results provided insight into how quercetin may inhibit or enhance growth of these specific microbes [4]. This strategy is particularly meaningful because it allows for the effect of quercetin to be determined independently and on individual strains of bacteria without the added factors that occur in a competitive environment. It is also important that the strains tested in this study are in fact representatives of a healthy human gut, and that each strain exhibits multiple unique properties [19–25]. However, evaluating the change in growth only partially describes how quercetin is able to effect the bacterial cell. Due to the genetic differences between *B.catenulatum, E.caccae,* and *R.gauvreauii*, it is possible that they respond to quercetin in the same way phenotypically, but do so through independent genetic pathways. Therefore, they would exhibit the same phenotype, yet maintain different patterns of gene expression which would be detectable at the mRNA level.

Recent advances in high-throughput sequencing technology allows for the sequencing and identification of mRNA at the single molecule level, without the use of probes, amplification or hybridization procedures [26]. This new technology is able to detect transcripts present at a low level, therefore more sensitive to small changes, produces less background and has a higher degree of reproducibility [26]. The application of single molecule sequencing to study bacterial gene expression has provided a wealth of information on bacterial gene regulation. This technology has been previously used to analyze the transcriptome of soil microbial communities [27], and mapped the transcriptome of *Actinobacillus pleuropneumoniae* [28], and methicillin resistant *Staphylococcus aureus* treated with ursolic acid and resveratrol [29].

In the current study, the effect of quercetin on *B.catenulatum, E.caccae,* and *R.gauvreauii* was evaluated through both phenotypic and genotypic analysis. The change in growth of these bacteria in response to different doses of quercetin was first documented over 24 h in order to generate growth curves. Images of each bacteria grown with or without quercetin were captured using Scanning Electron Microscopy (SEM) technology to check for any morphological differences. The genetic expression profiles for each strain grown with or without the addition of quercetin were then generated using true single molecule RNA sequencing via Helicos technology. The genotypic effect of quercetin on *B.catenulatum, E.caccae,* and *R.gauvreauii* was determined by comparing the expression profiles of quercetin-treated bacteria to the control. The results of this analysis demonstrated that each strain responded to quercetin through separate means, indicating that while the phenotypic response may be the same, there is a difference at the genetic level. This study provides a more in depth examination on how quercetin may affect commensal gut microbes, and how the use of advanced RNA sequencing technology can be applied to the study of the gut microbiota.

2. Materials and methods

2.1. Culturing of anaerobic bacteria and broth preparation

Bacteria were ordered from the company Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Germany. The following strains were ordered and used for all experiments: Freeze Dried ampoule of 19829, Type strain; *Ruminococcus gauvreauii* CCRI-16110 (*R. gauvreauii*), Freeze Dried ampoule of 19114, Type strain; *Enterococcus caccae* SS-1777 (*E.caccae*), Freeze Dried ampoule of 16992, Type strain; *Bifidobacterium catenulatum* B669 (*B.catenulatum*). Frozen aliquots of bacteria were recovered through inoculation in strain specific broth, described below, and grown overnight in the anaerobic chamber at 37 °C. Each bacterial strain was cultured and grown overnight sequentially at least twice prior to use in order to ensure recovery from freezing.

All anaerobic broth was autoclaved under pressure at 120 °C for 30 min. After autoclaving, the broth was boiled under negative pressure using nitrogen gas for 10 min to remove any oxygen from the solution [30,31]. The broth was then placed inside a Bactron anaerobic chamber to cool overnight in oxygen free conditions. All anaerobic broths were stored at room temperature in oxygen free conditions until use and made fresh every two to three weeks. Each strain was cultured and grown in the broth recommended by DSMZ, all ingredients for which were purchased from Sigma-Aldrich. *R. gauvreauii* was cultured in Peptone Yeast Glucose broth (modified), *B. catenulatum* was cultured in Bifidobacterium medium and *E. caccae* was cultured in Trypticase Soy Yeast Extract medium [4].

2.2. The polyphenol quercetin

The polyphenol quercetin (Q4951-100G) was purchased from the company Sigma-Aldrich and dissolved in DMSO to make a working stock solution (freshly made for each experiment). For all testing, the DMSO stock solution of quercetin was added to the anaerobic broth at a volume of 10 μL DMSO stock solution per 5 mL of anaerobic broth (all final cultures contained 1% DMSO). Quercetin was tested at a final concentration of 12.5, 25, 50, and 75 μg/mL for each bacterial strain. For each concentration tested the polyphenol added to the anaerobic broth without addition of bacteria was used as a negative control and bacteria cultured in anaerobic broth containing DMSO without polyphenol was used as a positive control.

2.3. The effect of quercetin on growth of R. gauvreauii, E. caccae, and B. catenulatum

All work for this experiment was performed in a Bactron anaerobic chamber to ensure oxygen free conditions. Each bacterial strain was maintained and experiments performed using their specific anaerobic broth as described above. Anaerobic broth was

Peptone Yeast Glucose Broth (modified); Final pH 7.2	
Ingredient	Amount in 1 L dd H_2O
Trypticase peptone	5.00 g
Peptone	5.00 g
Yeast extract	10.00 g
Beef extract	5.00 g
Glucose	5.00 g
K_2HPO_4	2.00 g
Tween80	1.00 mL
Cysteine-HCl x H_2O	0.50 g
Resazurin	1.00 mg
Salt solution	40 mL
Haemin solution	10 mL
Vitamin K solution	0.20 mL

Bifidobacterium medium; Final pH 6.8	
Ingredient	Amount in 1 L dd H_2O
Casein peptone tryptic digest	10.00 g
Yeast extract	5.00 g
Meat extract	5.00 g
Bacto Soytone	5.00 g
Glucose	10.00 g
K2HPO4	2.00 g
MgSO4 x 7 H20	0.20 g
Tween80	1.00 mL
NaCl	5.00 g
Cysteine-HCl x H2O	0.50 g
Resazurin	1.00 mg
Salt solution	40 mL

Salt solution	
Ingredient	Amount in 1 L dd H_2O
$CaCl_2$ x 2 H_2O	0.25 g
$MgSO_4$ x 7 H_2O	0.50 g
$K2HPO_4$	1.00 g
$KH2PO_4$	1.00 g
$NaHCO_3$	10.00 g
NaCl	2.00 g

Haemin solution	
Ingredient	Amount in 100 mL dd H_2O
Haemin	50 mg dissolved in 1 mL of 1N NaOH

Trypticase Soy Yeast Extract Medium; Final pH 7.1	
Ingredient	Amount in 1 L dd H_2O
Trypticase Soy broth	30.00 g
Yeast extract	3.00 g

aliquoted into hungate tubes prior to starting an experiment, 5 mL per tube. The tubes were sealed with a rubber septa and screw cap lid, and stored at room temperature in the anaerobic chamber until needed. (The hungate tubes, rubber septa and screw cap lids were ordered from Chemglass.) All three strains of anaerobic bacteria were grown overnight (16 h) at 37 °C under anaerobic conditions in 5 mL of strain specific anaerobic broth.

To begin the experiment, each hungate tube containing pre-aliquoted broth was injected with DMSO containing quercetin to the desired final concentration (1 mL needle and 25 gauge syringe). The culture of bacteria grown overnight was diluted in their specific broth to 0.5 McFarland units (MU) over the broth only read. Each 5 mL hungate tube containing the appropriate anaerobic broth and the desired concentration of polyphenol was injected with 100 μL of this culture using a 1 mL needle and a 25 gauge syringe. Each hungate tube was briefly vortexed after injection to ensure proper distribution and the MUs for each culture were determined using a densitometer (time 0 read). The cultures were then placed into the anaerobic incubator set to 37 °C. Each culture was removed from the incubator at 4, 8, 12, and 24 h post inoculation, briefly vortexed, and the MUs were measured using a densitometer. Each concentration of quercetin tested was considered a set and consisted of six hungate tubes of broth containing the desired polyphenol. Three tubes were designated as a negative control, consisting of broth containing the desired concentration of polyphenol only. The other three tubes were designated as the experimental group, consisting of broth containing polyphenol which were also inoculated with bacteria.

The McFarland readings from each group were adjusted by subtracting the broth control read. The adjusted numbers were plotted in a growth curve as the change in MU over time. The percent of control was calculated by dividing the MU of the experimental group by the MU of the control and multiplying by 100. Time points 0, 4, and 8 h post inoculation are not considered for percent of control for the strains *R. gauvreauii* and *B. catenulatum*, and time points 0 and 4 h post inoculation are not considered for percent of control for the strain *E. caccae*. This is because the MU readings were too low at these time points for this type of analysis. For each time point a 2-tailed, student *t*-test was run to determine if the difference between the control and the experimental groups was statistically significant.

2.4. RNA extraction and RNA sequencing

The effects of quercetin on bacterial gene expression and regulation were determined through mRNA analysis via Single Molecule RNA sequencing using Helicos technology (SeqLL, Boston, MA). Total RNA was extracted from bacteria grown in the presence of either quercetin or DMSO only, at the concentration of polyphenol that was determined to have the most effect. *R.gauvreauii* was cultured in the presence of 50 μg/mL of quercetin or 1% DMSO for 16 h. *B. catenulatum* was cultured in the presence of 50 μg/mL of quercetin or 1% DMSO for 8 h. *E. caccae* was cultured in the presence of 25 μg/mL quercetin or 1% DMSO for 10 h. After growth, 15 mL of these cultures were added to a 50 mL falcon tube and spun for 10 min at 5000 g to pellet the bacteria. Post centrifugation, the supernatant was discarded, 1 mL of Trizol reagent was added, and the pellet/Trizol mix was vortexed to ensure homogenization. These samples were then frozen and stored at −80 °C until needed.

Prior to RNA sequencing, an RNA extraction was performed using the following Zymo Direct-zol RNA Miniprep protocol: 250 μL of Trizol was added to 100 μL of each sample stored in Trizol (described above). Next, 350 μL of pure ethanol was added directly to each sample and homogenized. The sample/ethanol mixture was then loaded into the Zymo-Spin II C column and centrifuged for 30 s at 16000 g. After centrifugation, the column was washed with 400 μL of RNA wash buffer and then centrifuged for another 30 s. Then, 80 μL of DNase I reaction mixture (5 μL DNAse I and 75 μL of DNA digestion buffer) was added and the sample incubated at 25 °C for 15 min. Next, 400 μL of Direct-zol RNA prewash was applied to the column, centrifuged for 30 s and the flow through was discarded. This step was repeated a second time. Then, 700 μL of RNA wash buffer was added to each column and centrifuged for 2 min, repeated twice. The column was transferred to an RNase free tube, 30 μL of RNase free water was added, and was centrifuged for 30 s. The final product was stored at −80 °C until needed.

2.5. RNA sequencing data analysis

Single molecule RNA sequencing was carried out in a Helicos sequencer by the company SeqLL (Boston, MA). In order to quantify the gene expression of each bacterial strain through its treatment, the full assembled genomes were downloaded from the National Center of Biological Information (NCBI http://www.ncbi.nlm.nih.gov/nuccore/NC_013198) [32]. Then, the reads of each sample

were mapped to their corresponding genome using UCLUST [33]. In order to increase the matching specificity, reads that were aligned to multiple locations were assigned to their corresponding best match. In this way, the genes are depicted by their number of unique reads for each sample. Due to the different levels of abundance through the samples, they were first normalized by their abundance and length using the RPKM [34] metric (reads per kilo base of mapped reads). Finally, the gene expression levels were computed by the log transformation of the RPMK abundances with respect to each control, log 2 (RPKM[sample]/RPKM[control]). For analytic purposes, genes with less than 50 reads total were not considered. The fold change in expression for each gene was determined through comparison of the experimental group (quercetin treated) to the control group (DMSO only treated). Any gene that had a higher number of reads compared to the DMSO only control was considered upregulated and, conversely, any gene that had a lower number of reads was considered downregulated.

2.6. Images of bacteria captured using SEM technology

Images of each bacterial strain treated with either quercetin or DMSO were captured using SEM technology to analyze cell morphology. To start, *R. gauvreauii* was cultured in 25 mL of Peptone Yeast Glucose broth in the presence of 50 μg/mL of quercetin or 1% DMSO for 16 h. *B. catenulatum* was cultured in 25 mL of Bifidobacterium broth in the presence of 50 μg/mL of quercetin or 1% DMSO for 8 h. *E. caccae* was cultured in 25 mL of Trypticase Soy Yeast Extract broth the presence of 25 μg/mL quercetin or 1% DMSO for 10 h.

After the growth period, the bacteria were spun down at 5 g for 5 min to condense the culture, and the supernatant was discarded. In the anaerobic chamber, the remaining pellet was vortexed and 20 μL of bacteria were pipetted onto an acetone cleaned 12 mm Micro-cover glass slide (Thermo Scientific, Portsmouth NH, USA). The bacteria were allowed to incubate on the slide for 30 min to allow for proper adhering. Afterwards, 50 μL of 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield PA, USA) was added to fix the cells for 30 min. The samples were then rinsed twice for 30 min each with 2–3 mL of the 0.1 M imidazole, (Electron Microscopy Sciences, Hatfield PA, USA), followed by 30 min intervals each of 50, 80, 90% ethanol (The Warner-Graham Company, Cockeysville, MD, USA), at 2–3 mL each. The samples were then washed and held three times with 2 mL of 100% ethanol before being critically point dried. The samples were stacked in a wire basket, separated by cloth, and placed in a *Critical Point Drying Apparatus*, (Denton Vacuum, Inc, Cherry Hill, NJ, USA), using liquid carbon dioxide (Welco Co, Allentown PA, USA) for approximately 20 min. The samples were mounted on stubs and sputter gold coated for 1 min (EMS 150R ES, EM Sciences, Hatfield, PA). Samples were then viewed with a FEI Quanta 200 F Scanning Electron Microscope, (Hillsboro, OR, USA) with an accelerating voltage of 10 KV in high vacuum mode.

3. Results

3.1. Effect of quercetin on the growth and morphology of *R. gauvreauii, B. catenulatum and E. caccae*

R. gauvreauii, B. catenulatum and *E. caccae* were cultured in strain specific broth containing different concentrations of quercetin. Overall growth of each culture was measured in MUs at 0, 4, 8, 12, and 24 h post inoculation using a densitometer. The phenotypic effects of quercetin on *R. gauvreauii*, *B. catenulatum* and *E. caccae* was examined by comparing the rate and pattern of growth for cultures treated with increasing concentrations of quercetin to the control group containing no polyphenol.

At zero and 24 h post inoculation there was no significant difference ($p > 0.05$) between the control and cultures of *R. gauvreauii* grown in the presence of quercetin at any concentration (Fig. 1A). At 8 h post inoculation, there was a significant increase in growth for *R. gauvreauii* treated with either 75, 50, or 25 μg/mL quercetin according to a 2-tailed, student *t*-test (Fig. 1A). However, the MU readings at this time point were markedly low: 0.40 MU for 75 μg/mL quercetin, 0.63 MU for 50 μg/mL quercetin, 0.33 MU for 25 μg/mL quercetin, and only 0.01 MU for the control, making them unreliable. Therefore, it is misleading to say this difference is actually significant. At 12 and 24 h post inoculation there was no statistical difference in growth between any concentration of quercetin and the control group. This is best illustrated in the figure showing percent of control, where growth ranges from 90.3%, for 75 μg/mL of quercetin to 106.5%, for 50 μg/mL of quercetin at the 12 h time point (Fig. 1B). Using SEM, whether or not quercetin had an effect on the overall morphology of *R. gauvreauii* was determined. There was no noticeable difference in size or cell shape for *R. gauvreauii* treated with 50 μg/mL of quercetin (Fig. 1C and D) compared to that of the control (Fig. 1E and F) at either 10,000 or 25,000 times magnification.

For *B. catenulatum*, the addition of quercetin produced no real inhibition of growth at 0, 4 and 8 h post inoculation (Fig. 2A). However, at 12 h post inoculation there was a significant reduction in growth of *B. catenulatum* treated with all concentrations of quercetin, except at the lowest concentration of 12.5 μg/mL. By 24 h post inoculation, there was a significant decrease in growth of *B. catenulatum* for all concentrations of quercetin tested (Fig. 2A). This suppression is best represented in the percent of control, where groups treated with quercetin ranged from 87.9% of the control for 25 μg/mL quercetin at the 12 h time point, to 95.5% of control for 12.5 μg/mL quercetin at the 12 h time point (Fig. 2B). At 24 h post inoculation, the MU readings for *B. catenulatum* were 9.13 MU for 75 μg/mL quercetin, 9.23 MU for 50 μg/mL quercetin, 9.3 for 25 μg/mL quercetin, 9.33 MU for 12.5 μg/mL quercetin, and 9.87 MU for no quercetin. These reading indicated that the observed inhibition of growth may be dose dependent. The effect of quercetin on cell morphology of *B. catenulatum* was determined using SEM technology. There was no observed change in size or cell shape for *B. catenulatum* treated with 50 μg/mL of quercetin (Fig. 2C and D) compared to that of the control (Fig. 2E and F) at either 10,000 or 25,000 times magnification.

For *E. caccae*, treatment with quercetin produced no significant difference at time zero (Fig. 3A). However, at 4, 8, 12, and 24 h post inoculation there was a statistically significant inhibition of growth for all concentrations, except for 12.5 μg/mL quercetin at 4 h and 50 μg/mL quercetin at 24 h post inoculation (Fig. 3A). At 24 h post inoculation the MU readings for *E. caccae* were 4.77 MU for 75 μg/mL quercetin, 5.03 MU for 50 μg/mL quercetin, 4.83 MU for 25 μg/mL quercetin, 4.77 MU for 12.5 μg/mL quercetin, and 5.4 MU for no quercetin. Because the severity of inhibition does not appear to correlate with the dose, it is rational to conclude that inhibition is not dose dependent. The suppression of growth due to quercetin is best presented by the percent control, where at 8 h post inoculation, all concentrations of quercetin had less than 90% growth, with the lowest being 80.3% for 75 μg/mL quercetin and the highest being 93.5% for 50 μg/mL quercetin (Fig. 3B). The percent of control increases at 12 and 24 h post inoculation, indicating that *E. caccae* was able to partially recover from the initial suppression. Using SEM technology, changes in cell morphology due to the addition of quercetin was examined. There was no observed change in size or cell shape for *E.caccae* treated with 50 μg/mL of quercetin (Fig. 3C and D) compared to that of the control (Fig. 3E and F) at either 10,000 or 25,000 times magnification.

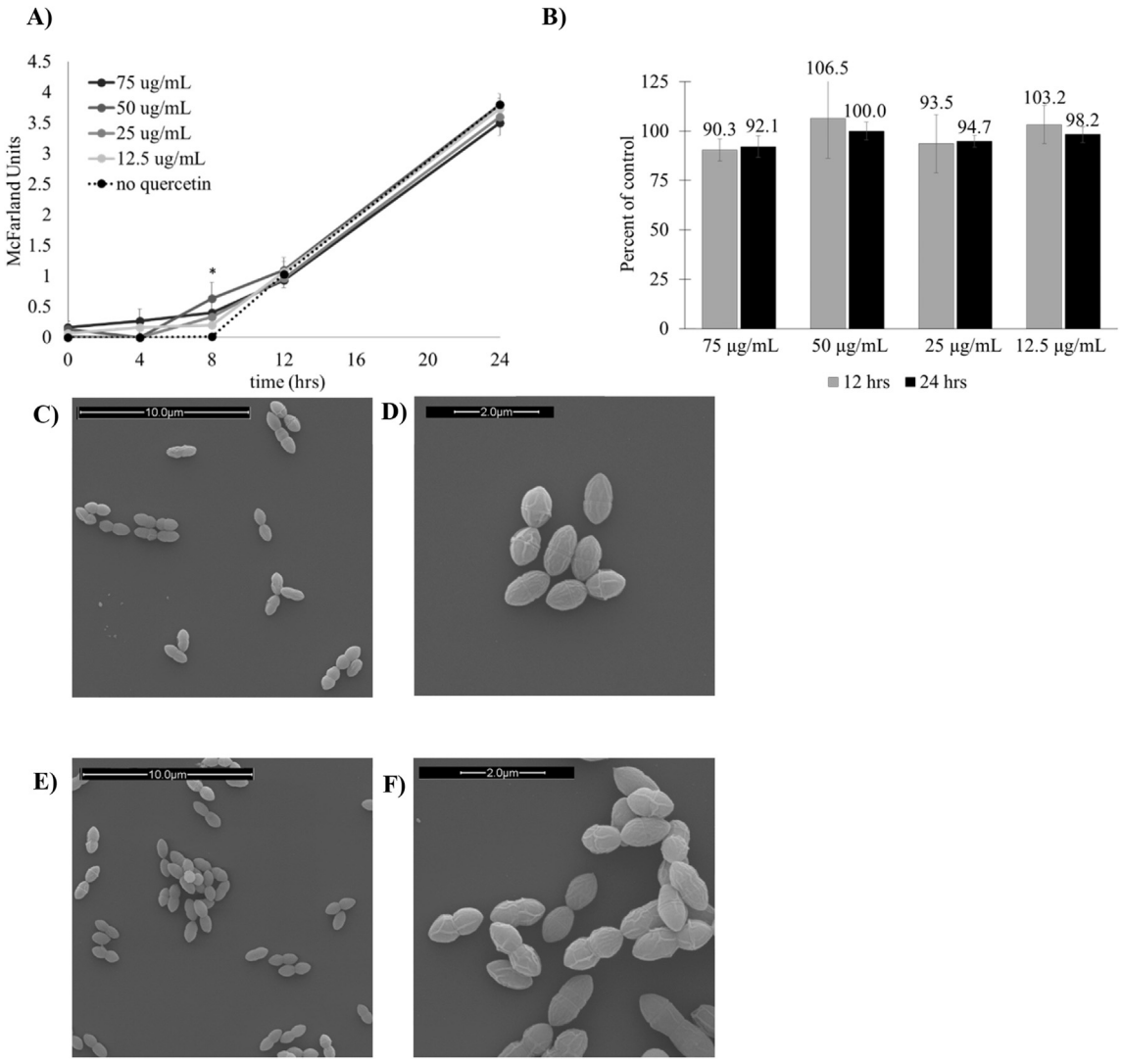

Fig. 1. The effect of quercetin on the growth and morphology of *R. gauvreauii*. *R. gauvreauii* was inoculated and grown in the presence of increasing concentrations of quercetin for 24 h. The MUs were measured using a densitometer at selected time points. The * mark indicates that the difference between the control and experimental groups was statistically significant at that time point, according to a 2-tailed, student *t*-test ($p < 0.05$). **A)** The 24 h growth curve of *R. gauvreauii* cultured in Peptone Yeast Glucose broth (modified) supplemented with increasing concentrations of quercetin. The dotted line represents bacteria grown without quercetin. Note that at the 8 h time point growth is significantly higher for all treated groups except 12.5 μg/mL, when compared to the control. **B)** The percent of control for each concentration of quercetin at 12 and 24 h post inoculation. **C)** Image of *R. gauvreauii* treated with 50 μg/mL of quercetin captured using SEM technology, magnified 10,000 times. **D)** SEM image of *R. gauvreauii* treated with 50 μg/mL quercetin, magnified 25,000 times. **E)** Image of *R. gauvreauii* treated with DMSO captured using SEM technology, magnified 10,000 times. **F)** Image of *R. gauvreauii* treated with DMSO captured using SEM technology, magnified 25,000 times.

3.2. Genetic expression profiles of Ruminococcus gauvreauii

The genetic expression profile of *R. gauvreauii* grown in the presence of 50 μg/mL of quercetin was assembled and compared to the expression profile of the control (DMSO treated). For *R. gauvreauii*, 209 individual genes were identified. Of these genes, only 26 were upregulated (12.4% of the total), 15 of which were hypothetical proteins, and only nine produced a greater than 1.25 fold increase in expression (Table 1A). The other 183 genes identified were downregulated compared to the control (87.6% of the total). Of the 183 downregulated genes, 32 produced a greater than 2.25 fold decrease in expression, 26 of which were hypothetical proteins (Table 1B).

3.3. Genetic expression profiles of Bifidobacterium catenulatum

The genetic expression profiles of *B. catenulatum* grown in the presence of 50 μg/mL of quercetin and compared to the expression profile of the control (DMSO treated). For *B. catenulatum* treated with quercetin, 745 genes were identified. Out of the 745 genes, only 170 were upregulated (22.8% of total), 67 of which were conserved hypothetical proteins and 14 of which were hypothetical proteins. Only 63 genes had a greater than 1.25 fold increase in expression, and only 19 had a greater than 1.5 fold increase in expression compared to the control (Table 2A). Of the genes identified a total of 575 were downregulated (77.2% of total), with 417 having a greater than 1.25 fold decrease in expression. There were 32 genes identified that had a greater than 2.5 fold decrease in expression, 13 of which were hypothetical or conserved hypothetical proteins (Table 2B).

3.4. Genetic expression profiles of Enterococcus caccae

The genetic expression profiles of *E. caccae* were cultured in the

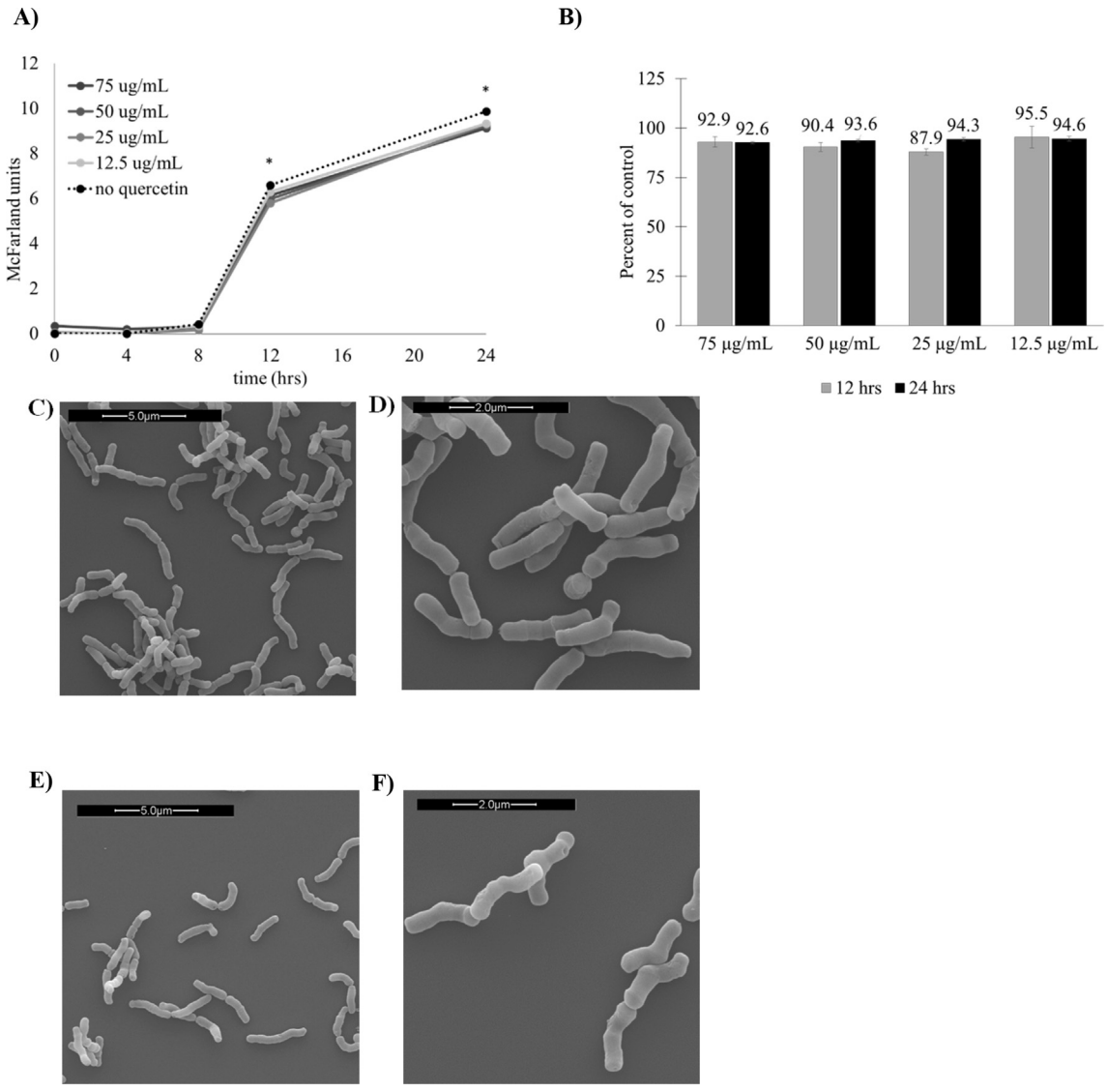

Fig. 2. The effect of quercetin on the growth of *B. catenulatum*. *B. catenulatum* was inoculated and grown in the presence of increasing concentrations of quercetin for 24 h. The MUs were measured using a densitometer at selected time points. The * mark indicates that the difference between the control and experimental groups was statistically significant at that time point, according to a 2-tailed, student *t*-test ($p < 0.05$). **A**) The 24 h growth curve of *B. catenulatum* cultured in Bifidobacterium medium supplemented with increasing concentrations of quercetin. The dotted line represents bacteria grown without quercetin. Note that at the 12 and 24 h time points, growth was significantly inhibited for all treated groups compared to the control except for 12.5 μg/mL at 12 h. **B**) The percent of control for each concentration of quercetin at 12 and 24 h post inoculation. **C)** Image of *B. catenulatum* treated with 50 μg/mL of quercetin captured using SEM technology, magnified 10,000 times. **D)** Image of *B. catenulatum* treated with 50 μg/mL quercetin captured using SEM technology, magnified 25,000 times. **E)** Image of *B. catenulatum* treated with DMSO captured using SEM technology, magnified 10,000 times. **F)** Image of *B. catenulatum* treated with DMSO captured using SEM technology, magnified 25,000 times.

presence of 25 μg/mL of quercetin and compared to the expression profile of the control (DMSO treated). For *E. caccae* treated with quercetin, 759 genes were identified. Of these genes, 413 were upregulated (54.4% of total), 149 of which were hypothetical proteins. Out of these upregulated genes, 48 produced a greater than 1.25 fold increase in expression and nine produced a greater than 1.5 fold increase in expression (Table 3A). There were 346 genes found to be downregulated in response to quercetin (45.6% of total), 139 of which were hypothetical proteins. Out of the downregulated genes, 78 had a greater than 1.25 fold decrease in expression, and 26 had a 1.5 fold decrease in expression compared to the control (Table 3B).

4. Discussion

Our results demonstrated that quercetin treatment had no substantial effect on the growth of *R. gauvreauii* (Fig. 1A and B). The only statistically significant effect was an increase in growth at the 8 h time point; however, this is likely to be artefact due to low MU readings. Interestingly, at a concentration of 75 μg/mL of quercetin, *R. gauvreauii* growth only reached 90.3% of control at 12 h and 92.1% of control at 24 h (Fig. 1B). While this difference was not statistically significant according to a 2-tailed, student *t*-test, it was perceptible, and indicated that the addition of quercetin may not be immaterial. Based on this observation, we hypothesized that even though there is no real phenotypic change, the effects of quercetin on *R. gauvreauii* would be evident at the genotypic level.

For *R. gauvreauii* treated with quercetin, 209 individual genes were identified and their levels of expression were compared to the DMSO only control. Of these 209 genes, only 25 were upregulated in response to quercetin, and only nine of these had a greater than 1.25 fold increase in expression (Table 1A). The five genes with the

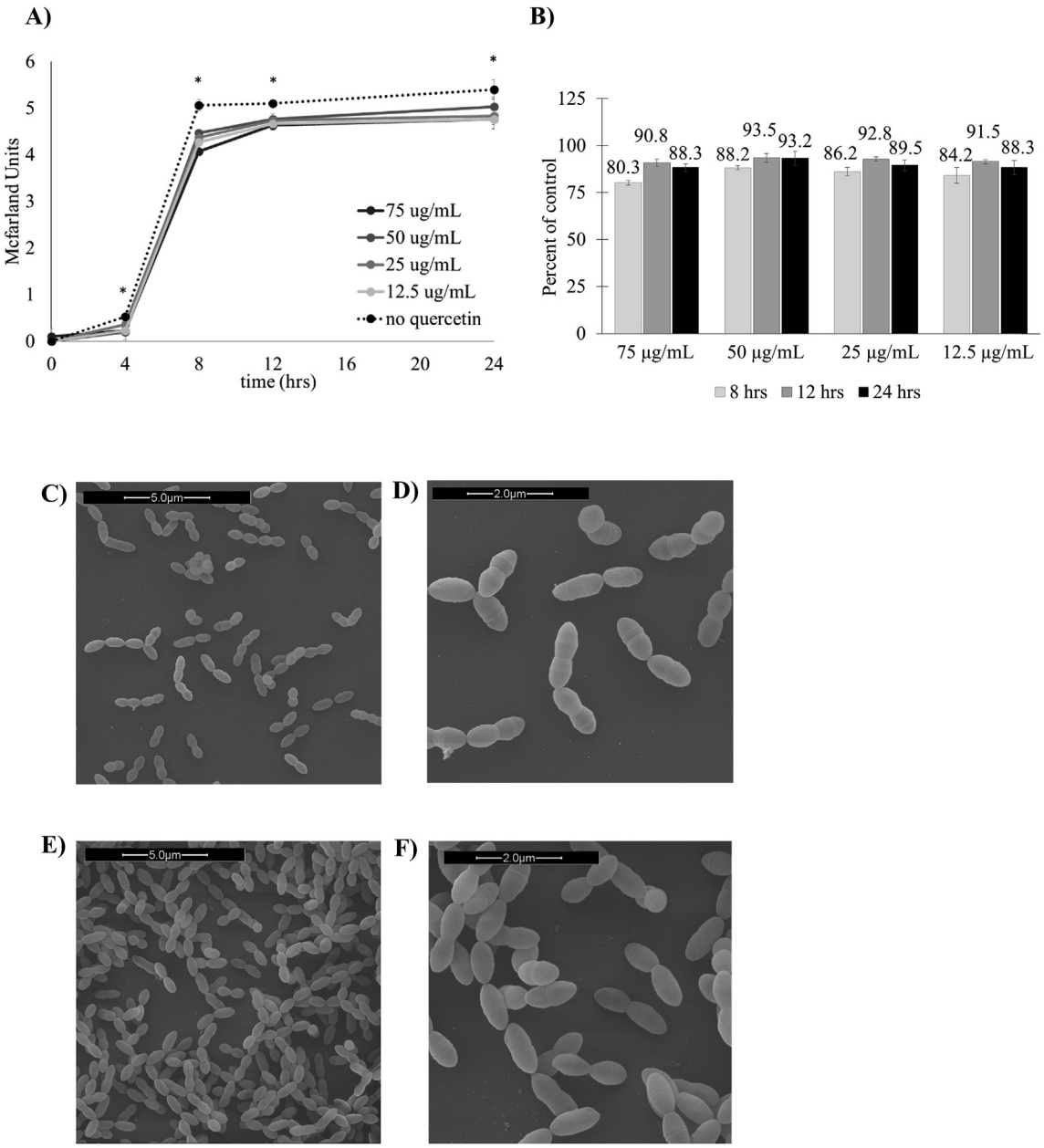

Fig. 3. The effect of quercetin on the growth of *E.caccae*. *E. caccae* was inoculated and grown in the presence of increasing concentrations of quercetin for 24 h. The MUs were measured using a densitometer at selected time points. The * mark indicates that the difference between the control and experimental groups was statistically significant at that time point, according to a 2-tailed, student *t*-test ($p < 0.05$). **A**) The 24 h growth curve of *E.caccae* cultured in Trypticase Soy Yeast Extract medium supplemented with increasing concentrations of quercetin. The dotted line represents bacteria grown without quercetin. Note that growth was significantly inhibited by all concentrations of quercetin at 4, 8, 12, and 24 h post inoculation compared to the control except for 50 µg/mL at 24 h. **B**) The percent of control for each concentration of quercetin at 8, 12, and 24 h post inoculation. **C)** Image of *E. caccae* treated with 25 µg/mL of quercetin captured using SEM technology, magnified 10,000 times. **D)** Image of *E. caccae* treated with 25 µg/mL quercetin captured using SEM technology, magnified 25,000 times. **E)** Image of *E. caccae* treated with DMSO captured using SEM technology, magnified 10,000 times. **F)** Image of *E. caccae* treated with DMSO captured using SEM technology, magnified 25,000 times.

highest increases in expression were all hypothetical proteins with an observed 1.5–2.0 fold change (Table 1A). Since they are listed as hypothetical proteins and their functions have not yet been defined, not much can be determined from this data. However, it is remarkable to find a group of them together. It is possible that they belong to a single system that has not yet been identified, or they may work together to perform a specific function in *R. gauvreauii*. It is also quite possible that they are unrelated.

There were only two other genes identified that had a greater than 1.25 fold increase in expression in response to quercetin: a peptide ABC transporter permease and a secondary thiamine-phosphate synthase, both with a fold increase of 1.4 (Table 1A). The ABC transporter permease is a key player in the ABC transporter system, a well-defined system of cellular transporters ubiquitous in bacteria [35]. The ABC transporter system is responsible for the transport of molecules into and out of the cell and is a contributing factor in antibiotic resistance [35,36]. The thiamine phosphate synthase is a critical component of thiamine (Vitamin B1) synthesis [37]. Thiamine is essential for metabolism and is crucial for all life forms, including *R. gauvreauii* [37]. However, since the increase in expression for both of these genes is marginal and they are not related, it is unclear whether this increase is due to

Table 1A
Ruminococcus gauvreauii genes upregulated in response to quercetin.

Gene ID	Description	Fold ▲	Description of function
H604_RS0101225	Hypothetical protein	2.0	Unknown
H604_RS22190	Hypothetical protein	1.9	Unknown
H604_RS19870	Hypothetical protein	1.5	Unknown
H604_RS0101030	Hypothetical protein	1.5	Unknown
H604_RS0100655	Hypothetical protein	1.5	Unknown
H604_RS0100880	Peptide ABC transporter permease	1.4	Molecular translocation
H604_RS0100950	Secondary thiamine-phosphate synthase	1.4	Thiamine biosynthesis
H604_RS0101195	Hypothetical protein	1.3	Unknown
H604_RS0100515	Hypothetical protein	1.3	Unknown

Table 1B
Ruminococcus gauvreauii genes downregulated in response to quercetin.

Gene ID	Description	Fold ▼	Description of function
H604_RS0100305	Purine-nucleoside phosphorylase	4.1	Purine synthesis
H604_RS0100530	Nucleotide exchange factor GrpE	3.5	Protein folding
H604_RS0100265	Phosphonoacetaldehyde hydrolase	3.3	Metabolism
H604_RS0100590	Hypothetical protein	3.2	Unknown
H604_RS0100195	Hypothetical protein	3.2	Unknown
H604_RS0100630	Hypothetical protein	2.9	Unknown
H604_RS0100225	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	2.9	Metabolism
H604_RS0100435	Magnesium chelatase	2.9	Metabolism
H604_RS0100645	Inositol 2-dehydrogenase	2.8	Metabolism
H604_RS0100755	DNA ligase (NAD(+)) LigA	2.8	DNA Replication
H604_RS0100130	Molecular chaperone DnaJ	2.8	Protein folding
H604_RS0100350	Iron transporter FeoA	2.8	Iron uptake
H604_RS0101105	Hypothetical protein	2.7	Unknown
H604_RS0100520	Molecular chaperone DnaJ	2.6	Protein folding
H604_RS19810	Hypothetical protein	2.6	Unknown
H604_RS0100810	Hypothetical protein	2.6	Unknown
H604_RS0100450	Methyltransferase	2.5	Methylation
H604_RS0100235	Hypothetical protein	2.5	Unknown
H604_RS0100410	Molecular chaperone GroEL	2.5	Protein folding
H604_RS0100180	Phosphoribosylaminoimidazolesuccinocarboxamide synthase	2.4	Purine synthesis
H604_RS0100525	Molecular chaperone DnaK	2.4	Protein folding
H604_RS0100025	Hypothetical protein	2.4	Unknown
H604_RS0100475	Holliday junction DNA helicase	2.4	DNA repair
H604_RS0100815	ABC transporter	2.4	Molecular translocation
H604_RS0101070	Oxidoreductase	2.4	Metabolism
H604_RS0100820	Ribokinase	2.4	Metabolism
H604_RS19825	Hypothetical protein	2.3	Unknown
H604_RS0100455	Hypothetical protein	2.3	Unknown
H604_RS0100945	SDR family oxidoreductase	2.3	Metabolism
H604_RS0100090	Inositol 2-dehydrogenase	2.3	Metabolism
H604_RS0100170	Adenylosuccinate lyase	2.3	Purine synthesis
H604_RS0101085	Hypothetical protein	2.3	Unknown

Table 2A
Bifidobacterium catenulatum genes upregulated in response to quercetin.

Gene ID	Description	Fold ▲	Description of function
BBCT_0715	Hypothetical protein	2.3	Unknown
BBCT_1466	Conserved hypothetical protein	2.1	Unknown
BBCT_0472	Hypothetical protein	2.0	Unknown
BBCT_0202	Conserved hypothetical protein	1.9	Unknown
BBCT_0743	Conserved hypothetical protein	1.8	Unknown
BBCT_0807	Hypothetical protein	1.7	Unknown
BBCT_0952	Conserved hypothetical protein	1.7	Unknown
BBCT_0948	Conserved hypothetical protein	1.7	Unknown
BBCT_0697	ABC transporter permease component	1.6	Molecular translocation
BBCT_1237	Putative lipoprotein signal peptidase	1.6	Protein secretion
BBCT_1357	Putative adenine glycosylase	1.6	DNA repair
BBCT_0629	Truncated conserved hypothetical protein	1.6	Unknown
BBCT_1709	Ribonuclease P	1.6	Transcription
BBCT_0642	ABC transporter ATP-binding component	1.5	Molecular translocation
BBCT_0076	Thiazole synthase	1.5	Thiamine biosynthesis
BBCT_0091	Transcriptional regulator	1.5	Transcription
BBCT_1210	Phospho-N-acetylmuramoyl-pentapeptide- transferase	1.5	Cell wall synthesis
BBCT_1642	Sugar ABC transporter permease component	1.5	Molecular translocation
BBCT_1113	Putative multidrug transport protein	1.5	Molecular translocation

Table 2B
Bifidobacterium catenulatum genes downregulated in response to quercetin.

Gene ID	Description	Fold ▼	Description of function
BBCT_1455	L-ribulose-5-phosphate 4-epimerase	5.3	Metabolism
BBCT_1640	Hypothetical protein	4.6	Unknown
BBCT_1163	Conserved hypothetical protein	4.4	Unknown
BBCT_0095	Putative glutaredoxin	4.0	Stress response
BBCT_1543	UDP-galactopyranose mutase	3.9	Cell wall synthesis
BBCT_1330	Transposase	3.7	Stress response
BBCT_1179	Nicotinate-nucleotide pyrophosphorylase	3.4	Metabolism
BBCT_0040	Hypothetical protein	3.3	Unknown
BBCT_0142	Putative UDP-galactopyranose mutase	3.3	Cell wall synthesis
BBCT_1080	Conserved hypothetical protein	3.2	Unknown
BBCT_1078	Conserved hypothetical protein	3.2	Unknown
BBCT_1062	DNA ligase	3.1	DNA repair
BBCT_1329	Putative transposase	3.1	Stress response
BBCT_0569	Conserved hypothetical protein	3.1	Unknown
BBCT_0098	Transcriptional regulator	3.0	Transcription
BBCT_0272	Conserved hypothetical protein	2.9	Unknown
BBCT_1117	Conserved hypothetical protein	2.8	Unknown
BBCT_0034	Putative phosphoprotein phosphatase	2.8	Metabolism
BBCT_1116	Deoxyuridine 5'-triphosphate nucleotidohydrolase	2.8	Thymidine synthesis
BBCT_1594	Chaperone GrpE	2.8	Protein folding
BBCT_0011	Hypothetical protein	2.6	Unknown
BBCT_0954	Conserved hypothetical protein	2.6	Unknown
BBCT_1033	Arginine repressor	2.6	Transcription
BBCT_1380	Conserved hypothetical protein	2.6	Unknown
BBCT_1369	Conserved hypothetical protein	2.6	Unknown
BBCT_0203	Conserved hypothetical protein	2.6	Unknown
BBCT_0008	Glutamate dehydrogenase	2.5	Metabolism
BBCT_0990	Ribosome recycling factor	2.5	Protein synthesis
BBCT_1001	Gamma-glutamyl phosphate reductase	2.5	Metabolism
BBCT_1563	Putative DNase	2.5	DNA repair
BBCT_1473	ATP synthase gamma subunit	2.5	ATP synthesis
BBCT_1011	Carbohydrate kinase	2.5	Metabolism

Table 3A
Enterococcus caccae genes upregulated in response to quercetin.

Gene ID	Description	Fold ▲	Description of function
UC7_RS13225	Hypothetical protein	2.0	Unknown
UC7_RS16330	Acyl carrier protein	1.7	Fatty acid synthesis
UC7_RS12845	RpiR family transcriptional regulator	1.6	Metabolism
UC7_RS13295	Cold-shock protein	1.6	Stress response
UC7_RS13975	Hypothetical protein	1.6	Unknown
UC7_RS16530	Hypothetical protein	1.5	Unknown
UC7_RS13120	Hypothetical protein	1.5	Unknown
UC7_RS16155	Hypothetical protein	1.5	Unknown
UC7_RS16200	4-oxalocrotonate tautomerase family enzyme	1.5	Metabolism

quercetin or to variation.

There were 32 genes identified which had a greater than 2.25 fold decrease in expression in response to quercetin (Table 1B). Notably, *R. gauvreauii* treated with quercetin resulted in the downregulation of three genes involved in purine synthesis; a purine-nucleoside phosphorylase gene with a 4.1 fold decrease, a phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR) synthase gene with a 2.4 fold decrease, and an adenylosuccinate lyase gene with a 2.4 fold decrease (Table 1B). The purine-nucleoside phosphorylase is ubiquitous in bacteria and involved in purine synthesis through the recovery of purine bases from both purineribo-nucleosides and dexoyribo-nucleosides [38]. Both the SAICAR synthase and the adenylosuccinate lyase are involved in de novo purine synthesis [39,40]. A downregulation of these genes indicates that either quercetin was directly inhibiting purine synthesis, or less purines were needed at that time. This would occur if there was a decrease in DNA replication or transcription, both of which typically require purine synthesis.

Also of interest was the downregulation of five genes involved in protein folding (Table 1B). These were the nucleotide exchange factor GrpE with a 3.5 fold decrease, two molecular chaperone DnaJ genes with a 2.8 and 2.6 fold decrease respectively, a molecular chaperone GroEL gene with a 2.5 decrease, and the molecular chaperone DnaK with a 2.4 fold decrease (Table 1B). These five genes code for protein chaperones, which ultimately function to maintain cellular proteostasis [41] through the prevention of protein aggregation and the refolding of misfolded proteins [42]. A downregulation of the chaperone pathway would indicate that there is either less protein being made or that misfolded proteins are being produced and not corrected.

Whether the ability of *R. gauvreauii* to maintain normal growth resulted from genetic upregulation or downregulation remains unclear. Nevertheless, there was a substantial disparity between the number of genes upregulated and the number downregulated. Of the 209 identified genes, 151 had a greater than 1.25 fold decrease in expression, which is over 16 times more than the number upregulated. This data indicated that *R. gauvreauii* may counter-act quercetin through a decrease in gene expression. The change in gene expression did not produce any changes in the size or shape of the bacteria, according to images captured using SEM

Table 3B
Enterococcus caccae genes downregulated in response to quercetin.

Gene ID	Description	Fold ▼	Description of function
UC7_RS14840	Hypothetical protein	1.9	Unknown
UC7_RS16020	Hypothetical protein	1.9	Unknown
UC7_RS15835	Peroxiredoxin	1.7	Stress response
UC7_RS14150	Serine hydrolase	1.7	Metabolism
UC7_RS13955	Protein-(glutamine-N5) methyltransferase_ release factor-specific	1.7	Methylation
UC7_RS11045	Hypothetical protein	1.6	Unknown
UC7_RS15880	Hypothetical protein	1.6	Unknown
UC7_RS13990	Hypothetical protein	1.6	Unknown
UC7_RS11475	Hypothetical protein	1.6	Unknown
UC7_RS13720	Hypothetical protein	1.6	Unknown
UC7_RS14195	PTS beta-glucoside transporter subunit EIIBCA	1.6	Sugar transport
UC7_RS13055	Amidophosphoribosyltransferase	1.6	Purine synthesis
UC7_RS11300	Hypothetical protein	1.5	Unknown
UC7_RS14385	Hypothetical protein	1.5	Unknown
UC7_RS11920	DNA topoisomerase IV subunit B	1.5	DNA replication
UC7_RS12280	Hypothetical protein	1.5	Unknown
UC7_RS11280	Hypothetical protein	1.5	Unknown
UC7_RS14715	Hypothetical protein	1.5	Unknown
UC7_RS15605	Hypothetical protein	1.5	Unknown
UC7_RS14845	Spermidine/putrescine ABC transporter spermidine/putrescine-binding protein	1.5	Molecular transport
UC7_RS13770	CAAX amino protease	1.5	Protein transport
UC7_RS15260	Hypothetical protein	1.5	Unknown
UC7_RS15895	D-alanyl-lipoteichoic acid biosynthesis protein DltD	1.5	Cell wall synthesis
UC7_RS13735	Hypothetical protein	1.5	Unknown
UC7_RS14935	Ribosomal RNA large subunit methyltransferase A	1.5	Methylation
UC7_RS11075	Holliday junction resolvase RecU	1.5	DNA repair

technology (Fig. 1C–F).

Ultimately, *R. gauvreauii* was able to maintain a normal phenotype under all concentrations of quercetin that were tested. Therefore, it is reasonable to conclude that any changes in genetic expression due to the addition of quercetin are both deliberate and necessary to sustain a normal growth pattern. These results indicate that in response to quercetin, *R. gauvreauii* upregulates a group of hypothetical proteins with unknown functions, and downregulates multiple genes responsible for metabolism, purine synthesis, and protein folding (Tables 1A and 1B).

Primary experimental results demonstrated that quercetin was able to statistically suppress growth of *B. catenulatum* at 12 and 24 h post inoculation (Fig. 2A and B). Although it was not a substantial reduction, with the percent ranging from 87.9 to 95.5% of control (Fig. 2B), and the detected phenotypic change was small, it provided evidence that the addition of quercetin is not inconsequential. Based on this conclusion, we hypothesized that the underlying source of this phenotypic change would be detectable at the genotypic level.

Treatment of *B. catenulatum* with quercetin resulted in a greater than 1.5 fold increase in expression for 19 individual genes (Table 2A). Markedly, the eight genes with the highest increase in expression were categorized as either hypothetical or conserved hypothetical proteins, ranging from a 1.7–2.3 fold increase (Table 2A). This is similar to the pattern of upregulation for *R. gauvreauii* treated with quercetin (Table 1A). It is possible that both sets of proteins are similar, but since the functions of these genes are unknown, no conclusion can be drawn.

Besides the hypothetical or conserved hypothetical proteins, there were four genes upregulated that are involved in molecular translocation (Table 2A). These four are: the ABC transporter permease component, with a 1.6 fold increase, as well as the ABC transporter ATP-binding component, the sugar ABC transporter permease component, and the putative multidrug transport protein, with a 1.5 fold increase in expression (Table 2A). As mentioned previously, the ABC transporter system is a well-defined system of cellular transporters, ubiquitous in bacteria, responsible for the transport of molecules into and out of the cell [35]. They are also involved in antibiotic resistance since they can be used by bacteria to efflux unwanted molecules from their interior [35,36]. It is possible *B. catenulatum* responds to quercetin by attempting to remove the substrate, which would explain the upregulation of these particular genes. Although each of these genes presented with a less than 2 fold increase, it may be significant that 21% of upregulated genes with over a 1.5 fold increase are identified as transporters.

There were 32 genes identified that had a greater than 2.5 fold decrease in expression in response to quercetin for *B. catenulatum.* Of these genes, six were associated with various pathways in metabolism, indicating that overall cellular function is decreased (Table 2B). This observation is further supported by the fact that the gene with the most reduction was the L-ribulose-5-phosphate 4-epimerase, which presented a 5.3 fold decrease in activity. The epimerase has been demonstrated to catalyze the interconversion of L-ribulose 5-phosphate and D-xylulose 5-phosphate, the last step of L-arabinose conversion [43,44]. L-arabinose is used by bacteria as a carbon source to produce energy [45]. Therefore, a decrease in L-ribulose-5-phosphate 4-epimerase would correlate to a decrease in catabolism and energy production.

Interestingly, there was also a decrease in the UDP-galactopyranose mutase and a putative UDP-galactopyranose mutase genes, with a 3.9 and 3.3 fold decrease respectively (Table 3B). The UDP-galactopyranose mutase is an essential enzyme that converts UDP-Gal*p* to UDP-Gal*f*, a main component of the bacterial cell wall and cell surface [46]. It would reason that if quercetin inhibits growth, less energy would be required with a decreased demand for cell wall proteins. It is also possible that quercetin limits cell wall synthesis, which reduces the ability of the bacterial cell to grow and, ultimately, to divide. Of note, the addition of quercetin did not affect morphology of *B. catenulatum* according to analysis of images captured using SEM technology (Fig. 2C–F).

In the end, growth of *B. catenulatum* was suppressed under all concentrations of quercetin tested at 24 h post inoculation. Therefore, it is rational to conclude that any changes in genetic expression due to the addition of quercetin, while deliberate, are insufficient to completely counteract the inhibitory effects. Our

results demonstrate that *B. catenulatum* responds to quercetin by increasing ABC transport, possibly in an attempt to remove the polyphenol from the cell, and the decrease of multiple metabolic pathways and cell wall synthesis. However, whether the change in regulation of these genes is the cause or the result of suppressed growth due to the addition of quercetin remains unclear.

Experimental results demonstrated that quercetin effectively inhibited growth of *E. caccae* at 4,8,12, and 24 h post inoculation. While suppression was only mild to moderate, ranging from 80.3 to 93.5% of control, it was continuous and *E. caccae* was unable to counteract the effect. Since there was an observed phenotypic effect of quercetin on *E. caccae,* it is hypothesized that the underlying cause of this phenotypic change would be detectable at the genotypic level.

For *E. caccae* treated with quercetin, there were only nine genes that had a greater than 1.5 fold increase in expression, five of which were identified as hypothetical proteins (Table 3A). Disregarding the five hypothetical proteins, of the four remaining, two of the upregulated genes were involved in metabolism, more specifically in energy production. The first gene, an RpiR family transcriptional regulator, was upregulated by 1.6 fold (Table 3A). Although this gene codes for a transcriptional element, it is listed as functioning in metabolism because this family of regulators is responsible for the transcription of genes involved in sugar catabolism [47]. The second gene, a 4-oxalocrotonate tautomerase family enzyme, is upregulated by 1.5 fold (Table 3A). This enzymes has the ability to use aromatic hydrocarbons as a source of carbon and energy [48]. An upregulation of two individual genes involved in energy production may explain why *E. caccae* is effected by quercetin but is still able to maintain growth.

There were 26 genes identified that were downregulated for *E. caccae* treated with quercetin. The gene with the largest decrease, disregarding the hypothetical proteins, was a peroxiredoxin (Table 3B). Peroxiredoxins are thiol-specific antioxidants that work via peroxidase activity, are also thought to be involved in signal transduction [49]. There was also a 1.7 fold decrease in the protein-(glutamine-N5) methyltransferase, a release factor-specific gene (Table 3B). Methyltransferases are enzymes that transfer methyl groups to substrates. It has been previously demonstrated that *N*5-methylation of the glutamine residue allows for the release of a polypeptide during translation [50]. It is possible that these pathways were downregulated to conserve energy, or that the observed decrease was due to a slower growth rate.

Interestingly, there was also a downregulation of two transporter genes, a result which was also found in *B. catenulatum* treated with quercetin. There is a 1.6 fold decrease in the PTS beta-glucoside transporter subunit EIIBCA and a 1.5 fold decrease in the spermidine/putrescine ABC transporter spermidine/putrescine-binding protein (Table 3B). The spermidine/putrescine ABC transporter spermidine/putrescine-binding protein is a system that transports both the essential polyamines spermidine and putrescine into the cell [51]. The PTS beta-glucoside transporter subunit EIIBCA is part of the carbohydrate phosphotransferase system (PTS). This is a well-defined system in bacteria that is responsible for the uptake of sugar and carbohydrates into the cell [52]. For *E. caccae*, it seems that there is an upregulation of catabolic pathways, yet a downregulation of sugar transport. This appears counter-intuitive, but it is possible that with an absence of substrate transport into the cell, pathways to use other sources of carbon for energy would need to be upregulated to maintain homeostasis.

Ultimately, the growth of *E. caccae* was suppressed due to the addition of quercetin. However, SEM analysis revealed no changes in cell morphology due to quercetin treatment (Fig. 3C–F). Therefore it is logical to say that any changes in the pattern of genetic expression due to the addition of quercetin were only able to partially counteract any negative effects, or were ineffectual. These results indicate that in response to quercetin, *E. caccae* upregulated genes responsible for energy production and metabolism, and downregulated pathways of stress response, translation, and sugar transport.

Interestingly, each of the three bacterial strains tested responded differently to quercetin phenotypically. The addition of quercetin did not prevent growth of *R. gauvreauii*, mildly suppressed the growth of *B. catenulatum,* and inhibited growth of *E. caccae* with mild to moderate results. Because each of the bacterial strains tested is unique and contains different genes and regulatory elements, changes in their genetic expression cannot legitimately be compared to each other. However, there are a few observations regarding the genetic response of these strains to quercetin that were noted.

Remarkably, all three strains had an upregulation of hypothetical, or conserved hypothetical proteins. For *R. gauvreauii,* they represented the top five genes upregulated, and for *B. catenulatum,* the top eight genes coded for hypothetical proteins. However, unlike *R. gauvreauii* and *B. catenulatum, E. caccae* did not have a group of multiple hypothetical proteins at the top of its list. It is possible that the absence of upregulated genes in this group was the reason *E. caccae* was more susceptible to quercetin. Since the function of the hypothetical genes is unknown, there is no way to determine if this is truly the case. However, it is an interesting observation.

It was also noted that both *R. gauvreauii* and *B. catenulatum* had an increase in an ABC transporter permease and upregulation of the thiamine biosynthesis pathway. Conversely, neither of these were increased by *E. caccae.* Furthermore, *R. gauvreauii* and *B. catenulatum* also presented a more severe downregulation of genes. For *R. gauvreauii* there was a 4.1 fold decrease in purine nucleoside phosphorylase and for *B. catenulatum* there was a 5.3 fold decrease in 1-ribulose-epimerase. However, for *E. caccae* the maximum downregulation was only 1.9 fold. It is possible that these differences in the pattern of gene regulation are the reason why *E. caccae* was unable to counteract quercetin to the same extent as *R. gauvreauii* and *B. catenulatum.*

5. Conclusion

For the first time, the effect of the polyphenol quercetin on the growth and genetic expression of three different commensal gut bacteria was documented. Interestingly, we found the growth patterns differed in response to quercetin, with varying degrees of inhibition observed. Through analysis of the resulting genetic expression, we were able to determine which genes were upregulated and downregulated for each bacteria. The pattern of gene expression was consistent with the observed phenotype and provides insight into the interaction between genetic regulation and growth. This is also a unique demonstration of how RNA single molecule sequencing can be used to study the gut microbiota.

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