

Analysis of Temporal Changes in Growth and Gene Expression for Commensal Gut Microbes in Response to the Polyphenol Naringenin

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ABSTRACT: In this study, the effect of the flavanone naringenin on the growth and genetic expression of the commensal gut microbes, *Ruminococcus gauvreauii*, *Bifidobacterium catenulatum*, and *Enterococcus caccae*, was analyzed. Analysis of growth curves revealed that *Ruminococcus gauvreauii* was unaffected by naringenin, *Bifidobacterium catenulatum* was slightly enhanced by naringenin, and *Enterococcus caccae* was severely inhibited by naringenin. Changes in genetic expression due to naringenin were determined using single-molecule RNA sequencing. Analysis revealed the following responses to naringenin: *Ruminococcus gauvreauii* upregulated genes involved in iron uptake; *Bifidobacterium catenulatum* upregulated genes involved in cellular metabolism, DNA repair and molecular transport, and downregulated genes involved in thymidine biosynthesis and metabolism; *Enterococcus caccae* upregulated pathways involved in transcription and protein transport and downregulated genes responsible for sugar transport and purine synthesis. For the first time, changes in growth and gene expression for commensal gut bacteria in response to naringenin were documented.

KEYWORDS: Naringenin, gut microbiota, single-molecule RNA sequencing

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Introduction

Naringenin is a plant polyphenol that belongs to the subgroup flavanone.¹ It is found in citrus fruits, predominantly grapefruit, and to some extent in tomatoes, tomato skin, and ketchup.^{1–3} Due to individual differences in diet, it is difficult to accurately determine the daily intake of naringenin. However, it is estimated that the average dietary intake of polyphenols is between 0.15 and 1.0 g/d.^{4,5} Similar to other polyphenols, the consumption of naringenin is associated with multiple health benefits.⁶ Naringenin has been demonstrated to have antioxidant activity,² and citrus flavanones have been demonstrated to have anti-inflammatory and anticancer properties.⁷ Naringenin also functions in regulating lipid metabolism,² and the consumption of orange juice containing flavanones has been indicated in reducing low-density lipoprotein cholesterol⁸ and increasing high-density lipoprotein concentrations.⁹

Naringenin is an aglycone; however, it is typically stored as a glycoside in plants, most commonly attached to either D-glucose or L-rhamnose.^{3,10} After ingestion, naringenin and its glycosides will pass through the small intestine unabsorbed and enter the large intestine, which is host to the gut microbiota.⁶ Bioavailability of naringenin depends on removal of the sugar groups by the gut microbiota to produce the aglycone naringenin, which can then

be absorbed in the large intestine.^{3,10,11} It has been previously demonstrated that the gut microbiota can not only remove the sugar moieties from the naringenin glycosides but also convert naringenin to phenolic acids through cleavage of the C-ring.^{1,10} However, although it is known that naringenin has both the opportunity and ability to interact with the gut microbiota, the effect of naringenin itself on the gut microbiota community remains unclear.

Research looking at the interaction of polyphenols and the gut microbiota has mainly focused on metabolism of polyphenols by the gut bacteria,¹² although there have been some studies demonstrating that polyphenols can affect bacterial viability by stimulating beneficial microbes and inhibiting pathogens.^{4,6} For example, the growth of pathogenic bacteria *Clostridium perfringens*, *Clostridium difficile*, and some *Bacteroides* spp was significantly inhibited by tea polyphenols.⁵ Several polyphenols have been demonstrated to suppress adhesion of the pathogen *Salmonella typhimurium* and enhance adhesion of the probiotic *Lactobacillus rhamnosus* to human gut cells.¹³ However, these studies predominantly analyzed the effect of polyphenols in combination, and not individually, making it difficult to elucidate which polyphenols are responsible for the effect.



Previously, a study of the effect of naringenin on the growth of human intestinal bacteria was performed.¹² However, this study only analyzed the effect of naringenin on bacterial growth without exploring the underlying cause. It is possible that naringenin affects bacteria at the genetic level, resulting in upregulation or downregulation of specific genes.^{14,15} In this case, the bacterial growth pattern may not change but the pattern of gene expression will become altered.^{14,15} Based on this hypothesis, to fully understand the effect of naringenin, both changes in growth and genetic expression need to be evaluated.

In this study, the effect of naringenin on the growth and genetic regulation of the commensal gut microbes *Ruminococcus gnavreaii*, *Bifidobacterium catenulatum*, and *Enterococcus caccae* was evaluated. These strains represent classical types of commensal microbes that would be found in the human large intestine and were all isolated from human feces.^{16–18} First, changes in growth due to the addition of naringenin were evaluated by culturing each strain with different concentrations of naringenin for 24 hours. During this time, the turbidity was measured at 0, 4, 8, 12, and 24 hours post inoculation using a densitometer to formulate the growth pattern of each species of bacteria. Second, gene expression profile of each species of bacteria treated with naringenin and the control group, with no naringenin added, was determined through single-molecule RNA sequencing via Helicos Technology and compared with identified changes in gene expression due to naringenin. This study provides evidence that the consumption of naringenin may result in modifications to the gut microbiota composition and gene expression. This effect may contribute to the overall health benefits associated with a diet high in naringenin.

Methods

Bacterial strains

All bacterial strains were ordered as freeze-dried ampoules from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Germany: type strain *Ruminococcus gnavreaii* CCRI-16110 (19829), type strain *Enterococcus caccae* SS-1777 (19114), and type strain *Bifidobacterium catenulatum* B669 (16992). Each species of bacteria was cultured in strain-specific broth as defined by DSMZ. Prior to being used for experimentation, the bacteria were revived from frozen stock by growing overnight (16 hours) in strain-specific broth under anaerobic conditions at 37°C. Bacteria were inoculated and cultured overnight at least 2 times in sequence prior to its use.

Anaerobic broth preparation

The strain-specific broth was prepared according to the composition supplied by DSMZ and autoclaved at 120°C for 30 minutes using the liquid cycle. Oxygen was then removed from the autoclaved broth by heating while under negative pressure (nitrogen gas) for 10 minutes. The broth was then

moved into a Bactron anaerobic chamber, where it was able to cool to room temperature while under anaerobic conditions. All broths were stored in the anaerobic chamber, at room temperature, for up to 3 weeks.

Peptone Yeast Glucose Broth (modified) was used to culture *R. gnavreaii* and comprised the following ingredients in a final volume of 1 L deionized/distilled water: trypticase peptone 5.00 g, peptone 5.00 g, yeast extract 10.00 g, beef extract 5.00 g, glucose 5.00 g, K₂HPO₄ 2.00 g, Tween 80 1.00 mL, cysteine-HCl×H₂O 0.50 g, resazurin 1.00 mg, salt solution 40 mL (salt solution composition: CaCl₂×2 H₂O 0.25 g, MgSO₄×7 H₂O 0.50 g, K₂HPO₄ 1.00 g, KH₂PO₄ 1.00 g, NaHCO₃ 10.00 g, NaCl 2.00 g, distilled water up to 1000.00 mL), hemin solution 10 mL (50 mg hemin in 1 mL 1 N NaOH; final volume of 100 mL using distilled water, stored at 4°C) and vitamin K₁ solution 200 μL (0.1 mL of vitamin K₁ in 20 mL 95% ethanol and filter sterilized, stored at 4°C). The final pH was adjusted to 7.2 using 10 M NaOH or 37% HCl. *Bifidobacterium* media was used to culture *B. catenulatum* and contains the following ingredients in a final volume of 1 L deionized/distilled water: 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, MgSO₄×7 H₂O 0.20 g, MnSO₄×H₂O 0.05 g, Tween 80 1.00 mL, NaCl 5.00 g, cysteine-HCl×H₂O 0.50 g, salt solution 40 mL (salt solution composition: CaCl₂×2 H₂O 0.25 g, MgSO₄×7 H₂O 0.50 g, K₂HPO₄ 1.00 g, KH₂PO₄ 1.00 g, NaHCO₃ 10.00 g, NaCl 2.00 g, distilled water up to 1000.00 mL), and resazurin 1.00 mg. The final pH was adjusted to 6.8 using 10 M NaOH or 37% HCl. Trypticase Soy Yeast Extract Medium was used to culture *Enterococcus caccae* and consisted of the following ingredients in a final volume of 1 L deionized/distilled water: trypticase soy broth 30.0 g and yeast extract 3.0 g. The final pH was adjusted to 7 to 7.2 using either 10 M NaOH or 37% HCl.

Preparation of naringenin

For each experiment, the polyphenol naringenin (W530098-500G; Sigma Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. Stock solutions were made to contain 100, 75, 50, and 25 mg/mL naringenin. For all testing, 10 μL DMSO stock solution of naringenin was added per 5 mL of anaerobic broth. This resulted in a final concentration of 200, 150, 100, and 50 μg/mL of naringenin in strain-specific broth containing 1% DMSO. For each experiment, the negative control consisted of naringenin added to the broth without bacteria, and the positive control consisted of bacteria cultured in anaerobic broth containing 1% DMSO without the addition of naringenin.

Evaluating changes in growth

To ensure oxygen-free conditions, all work was performed using a Bactron anaerobic chamber. To test the effects of

naringenin on each bacterial strain, the following steps were performed, in the manner described previously^{14,15}: to begin, strain-specific anaerobic broth was aliquoted into hungate tubes at a volume of 5 mL per tube.

Each individual hungate tube was sealed using a rubber septa and screw cap (Chemglass, Vineland, NJ, USA) and then stored at room temperature in the anaerobic chamber.

Each bacterial strain was inoculated the night before beginning each experiment and grown to confluency at 37°C. To start the experiment, the DMSO stock solution of naringenin was injected into the hungate tubes containing the aliquoted broth (1 mL needle; 25 gauge syringe). Next, the confluent culture of bacteria was diluted, in the broth specific to that type of bacteria, to 0.5 McFarland units (MU) and 100 µL of this culture was injected into each 5-mL hungate tube (1-mL needle; 25-gauge syringe). To ensure proper distribution after injection, each hungate tube was briefly vortexed and a densitometer was used to determine the MU (time 0 read). The hungate tubes were then incubated at 37°C in an anaerobic chamber. Finally, at 4, 8, 12, and 24 hours post inoculation a densitometer was used to determine the MU for each hungate tube. For each concentration of naringenin tested, 6 hungate tubes of broth containing the desired polyphenol were included. Three of these tubes were used as the negative control and were not inoculated with bacteria. The other 3 tubes were designated as the experimental group and were inoculated with bacteria. Changes in growth were considered statistically significance when $P \leq .05$, as determined using a 2-tailed, Student *t* test.

Evaluating gene expression

RNA extraction. Total RNA was extracted from bacteria cultured with naringenin or DMSO only, at the concentration determined to have the most influence on growth pattern. *Ruminococcus gawvreauii* was cultured in the presence of 200 µg/mL of naringenin and 1% DMSO for 16 hours. *Bifidobacterium catenulatum* was cultured in the presence of 200 µg/mL of naringenin and 1% DMSO for 8 hours. *Enterococcus caccae* was cultured in the presence of 50 µg/mL naringenin and 1% DMSO for 10 hours. After culturing for the determined amount of time, 15 mL of each were centrifuged at 5000g for 10 minutes. The bacterial pellet was then resuspended in 1 mL Trizol and stored at -80°C.

To extract total RNA, the following Zymo Direct-zol RNA Miniprep protocol as previously described^{14,15}: 250 µL of Trizol was added to 100 µL of sample followed by 250 µL of pure ethanol. This mixture was vortexed to homogenize. The entire mixture was loaded onto a Zymo-Spin II C column, centrifuged for 30 seconds at 16 000g, washed with 400 µL of RNA wash buffer, and then centrifuged again for 30 seconds. About 80 µL of DNase I reaction mixture (5 µL DNase I and 75 µL of DNA digestion buffer) was added and the column was incubated for 15 minutes at room temperature. After incubation,

400 µL of Direct-zol RNA prewash was added to the column and then the column was centrifuged for 30 seconds. The flow through was discarded, and this wash step was repeated. About 700 µL of RNA wash buffer was then added to the column and then the column was centrifuged for 2 minutes. This step was repeated twice. The total RNA was then eluted from the column by adding 30 µL of RNase (ribonuclease)-free water to the column and centrifuged for 30 seconds. The final product was stored at -80°C until needed.

RNA sequencing and data analysis. Single-molecule RNA sequencing was carried out using a Helicos sequencer by SeqLL (Boston, MA, USA) and the results were used to generate gene expression profiles, as previously described.^{14,15} The gene expression for each species of bacteria treated with either DMSO or naringenin was quantified following these steps. First, the fully assembled genomes were downloaded from the National Center of Biological Information (NCBI; <https://www.ncbi.nlm.nih.gov/nucleotide/NC013198>).¹⁹ Next, using UCLUST,²⁰ reads were mapped to their corresponding genomes. Any reads aligning to multiple locations were assigned to their corresponding best match so that the genes are presented by their number of unique reads. Then, using RPKM²¹ metrics, the reads were normalized according to abundance and length. Changes in gene expression were determined through the comparison of the experimental group (naringenin treated) with the control group (DMSO treated). Any gene that had a statistically significant higher number of reads compared with the DMSO-only control was considered upregulated and, conversely, any gene that had a lower number of reads was considered downregulated.

Results

Effects of naringenin on the growth and genetic regulation of *R. gawvreauii*

The phenotypic effect of naringenin on *R. gawvreauii* was determined by comparing the rate and pattern of growth for cultures treated with different concentrations of naringenin to the control group, which had no naringenin. There was a recorded enhancement of growth for *R. gawvreauii* at 12 hours post inoculation for all concentrations of naringenin tested, although this enhancement did not reach statistical significance (Figure 1A). This is also demonstrated in the percent of control (Figure 1A). This is also demonstrated in the percent of control, where at 12 hours post inoculation, culture density was greater than 100% for all concentrations of naringenin (Figure 1B). In fact, *R. gawvreauii* treated with 150 µg/mL naringenin resulted in 127.5% of growth when compared with the control at 12 hours and 105.9% of growth when compared with the control at 24 hours post inoculation (Figure 1B). Although these differences were not statistically significant, they were evident and indicated that the addition of naringenin may not be immaterial. Based on this supposition, it was hypothesized that even though there was no significant change in phenotype,

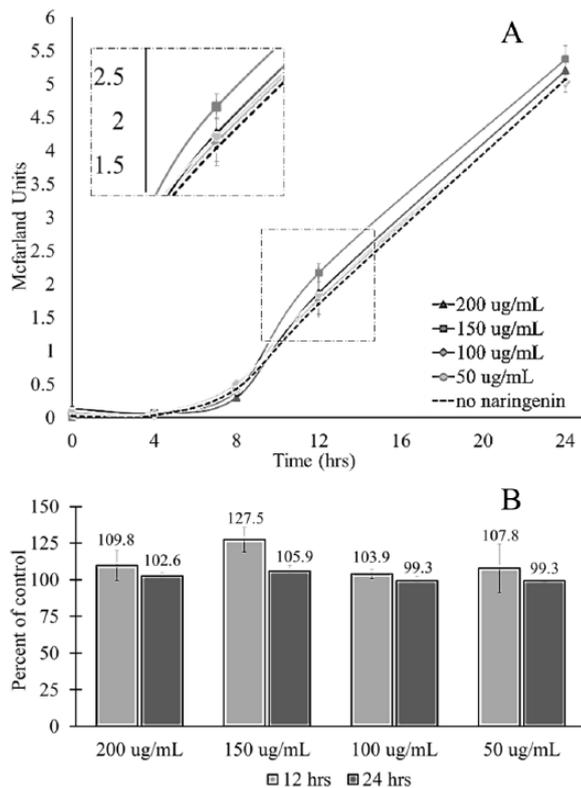


Figure 1. The effect of naringenin on the growth of *Ruminococcus gauvreauii*. *Ruminococcus gauvreauii* was cultured for 24 hours in the presence of increasing concentrations of naringenin. A densitometer was used to measure the McFarland units at designated time points. (A) The 24-hour growth curve of *R. gauvreauii* supplemented with increasing concentrations of naringenin. The dotted line represents bacteria grown without naringenin. (B) The percent of control for *R. gauvreauii* cultured in each concentration of naringenin at 12 and 24 hours post inoculation.

the effects of naringenin on *R. gauvreauii* may be evident in the pattern of gene expression.

The gene expression profile of *R. gauvreauii* grown in the presence of 200 $\mu\text{g}/\text{mL}$ of naringenin was assembled and compared with the expression profile of the control, no naringenin added (Tables 1 and 2). For *R. gauvreauii* treated with naringenin, 210 separate genes were identified. Out of the 210 genes, only 12 were found to have a greater than 1.25-fold increase in expression (Table 1) and only 6 produced a greater than 1.25-fold decrease in expression (Table 2). For brevity, only genes that had a greater than 1.25-fold change in expression were considered in the discussion.

Effects of naringenin on the growth and genetic regulation of *B. catenulatum*

The rate and pattern of growth for *B. catenulatum* treated with different concentrations of naringenin were compared with a control group, which had no naringenin. For *B. catenulatum*, the addition of naringenin produced no significant difference in growth at 0, 4, 8 and 24 hours post inoculation (Figure 2A). However, at 12 hours post inoculation, there was a significant increase in growth for *B. catenulatum* treated with all concentrations of

naringenin (Figure 2A). This is best demonstrated in the percent of control, where at 12 hours post inoculation, *B. catenulatum* treated with naringenin produced growth greater than 100% of the control (Figure 2B). Although the observed increase in growth for *B. catenulatum* treated with naringenin was significant, it was not extensive, only ranging from 106.4% to 109.4% of control (Figure 2B). There was also no distinct connection observed between an increase in dose and culture density, indicating that the observed enhancement is not dose dependent (Figure 2A and B). Although the detected phenotypic change of *B. catenulatum* due to naringenin was small, it provided evidence that this polyphenol does exert an effect on *B. catenulatum*. Based on this conclusion, it was hypothesized that the underlying cause of this phenotypic change could be determined by analyzing changes to gene expression.

To elucidate the genotypic effect of naringenin on *B. catenulatum*, the gene expression profile of *B. catenulatum* grown in the presence of 200 $\mu\text{g}/\text{mL}$ of naringenin was compared with the expression profile of the control (Tables 3 and 4). A total of 745 genes were identified, 108 genes produced over a 1.25-fold increase in expression, with 38 producing over a 1.5-fold increase in expression (Table 3). Of the genes identified, 44 produced a greater than 2.0-fold decrease in expression, and 17 had a greater than 2.5-fold decrease in expression (Table 4). For concision, only genes with a greater than 1.5-fold increase and 2.5-fold decrease in gene expression were considered for discussion.

Effects of naringenin on the growth and genetic regulation of *E. caccae*

The pattern and rate of growth for *E. caccae* cultured with increasing concentrations of naringenin was compared with *E. caccae* cultured with no naringenin (Figure 3). For *E. caccae*, at 4, 8, 12, and 24 hours post inoculation, growth was severely and statistically inhibited at all concentrations of naringenin examined (Figure 3A). At 24 hours post inoculation compared with the control, treatment with 200 $\mu\text{g}/\text{mL}$ naringenin produced only 49.3% growth, treatment with 150 $\mu\text{g}/\text{mL}$ naringenin resulted in 48.7% growth, treatment with 100 $\mu\text{g}/\text{mL}$ naringenin produced 60.5% growth, and treatment with 50 $\mu\text{g}/\text{mL}$ of naringenin resulted in 75.0% growth (Figure 3B). These results indicate that the inhibition of growth is dose dependent and that while suppression of growth is severe, *E. caccae* is able to partially recover from this inhibition over time.

Not only did naringenin affect the rate of growth but it also changed the pattern of growth for *E. caccae*. Without naringenin, the exponential growth phase occurred between 4 and 8 hours post inoculation, with growth leveling off at 12 hours post inoculation with subsequent entrance into the stationary phase (Figure 3A). The addition of naringenin suppressed entrance into the exponential phase until 8 hours post inoculation for 50 $\mu\text{g}/\text{mL}$ naringenin (Figure 3A). However, for *E. caccae* treated with 100, 150, or 200 $\mu\text{g}/\text{mL}$ naringenin, there was

Table 1. Genes upregulated by *Ruminococcus gausvreauii* in response to naringenin.

GENE ID	GENE DESCRIPTION	FOLD↑	FUNCTION
H604_RS0100855	Hypothetical protein	1.6	Unknown
H604_RS0100345	Iron transporter FeoA	1.4	Iron uptake
H604_RS0100045	Hypothetical protein	1.4	Unknown
H604_RS0100920	Proton-coupled thiamine transporter YuaJ	1.3	Thiamine transport
H604_RS0100110	Ribose ABC transporter permease	1.3	Molecular translocation
H604_RS0100455	Hypothetical protein	1.3	Unknown
H604_RS19840	Hypothetical protein	1.3	Unknown
H604_RS0100765	Hypothetical protein	1.3	Unknown
H604_RS0100350	Iron transporter FeoA	1.3	Iron uptake
H604_RS0100290	ATP-binding protein	1.3	Molecular translocation
H604_RS0100260	Hypothetical protein	1.3	Unknown
H604_RS0101200	Phosphatidic acid phosphatase	1.3	Metabolism

Table 2. Genes downregulated by *Ruminococcus gausvreauii* in response to naringenin.

GENE ID	GENE DESCRIPTION	FOLD↓	FUNCTION
H604_RS0100940	Hypothetical protein	1.4	Unknown
H604_RS0100620	Nucleotidyltransferase	1.3	DNA synthesis/repair
H604_RS0101055	D-ribose transporter ATP-binding protein	1.3	Molecular translocation
H604_RS0100980	Hypothetical protein	1.3	Unknown
H604_RS0100360	Hypothetical protein	1.3	Unknown
H604_RS0100485	tRNA (N(6)-L-threonylcarbamoyladenosine(37)-C(2))-methylthiotransferase MtaB	1.3	Translation

no obvious entrance into either exponential or stationary phase (Figure 3A). Based on these results, it was hypothesized that the observed change in phenotype of *E. caccae* in response to naringenin would be apparent at the genotypic level.

The gene expression profile of *E. caccae* cultured in the presence of 50 µg/mL of naringenin was assembled and compared with the expression profile of the control. For *E. caccae* treated with naringenin, 759 separate genes were identified (Tables 5 and 6). Of the 759 genes, 343 were upregulated (45.2% of total), of which 132 were hypothetical proteins. A total of 79 genes produced a greater than 1.25-fold increase in expression, with 28 producing a greater than 1.5-fold increase in expression compared with the control (Table 5). Out of the 759 genes, 416 were downregulated (54.8% of total), of which 156 were hypothetical proteins. Of the downregulated genes, 145 presented a greater than 1.25-fold decrease in expression and 49 genes produced a greater than 1.5-fold decrease in expression compared with the control (Table 6). For brevity, only genes with a greater than 1.5-fold increase and 2.5-fold decrease in gene expression were considered for discussion.

Discussion

Analysis of the rate and pattern of growth for *R. gausvreauii*, *B. catenulatum*, and *E. caccae* revealed that the addition of naringenin variably affected bacterial phenotype. For *R. gausvreauii*, treatment with naringenin resulted in no significant changes in phenotype, whereas for *B. catenulatum*, treatment resulted in an increase in growth at 12 hours post inoculation. For *E. caccae*, naringenin dramatically inhibited growth at 4, 8, 12, and 24 hours post inoculation and altered the pattern of growth by suppressing entrance into exponential phase. Although *R. gausvreauii*, *B. catenulatum*, and *E. caccae* were all exposed to the same amount of naringenin for the same length of time, the outcomes were incongruous. It was hypothesized that these differences in observed phenotype would be detected in the pattern of gene expression.

Analysis of the gene expression profiles for *R. gausvreauii* revealed that the addition of naringenin produced only minimal changes (Tables 1 and 2). There was only one gene identified with a greater than 1.5-fold increase in expression; unfortunately, this gene codes for a hypothetical protein and

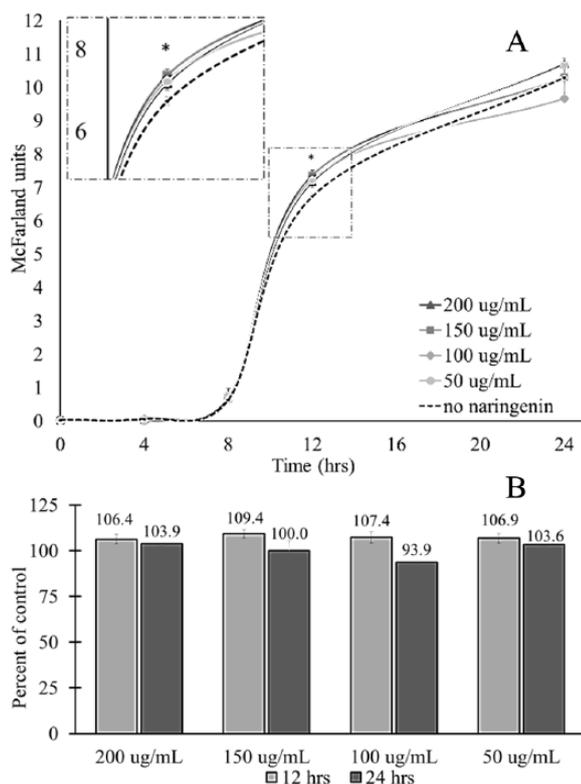


Figure 2. The effect of naringenin on the growth of *Bifidobacterium catenulatum*. *Bifidobacterium catenulatum* was cultured for 24 hours in the presence of increasing concentrations of naringenin. A densitometer was used to measure the McFarland units at designated time points. The * mark indicates that there was a statistically significant difference between the control and experimental groups at that time point, according to a 2-tailed, Student *t* test ($P < .05$). (A) The 24-hour growth curve of *B. catenulatum* supplemented with increasing concentrations of naringenin. The dotted line represents bacteria grown without naringenin. (B) The percent of control for *B. catenulatum* cultured in each concentration of naringenin at 12 and 24 hours post inoculation.

therefore its function is unknown (Table 1). Interestingly, the addition of naringenin resulted in the upregulation of 2 genes identified as iron transporter FeoA genes, with a 1.4- and 1.3-fold increase (Table 1). The FeoA gene is part of the Feo system, a ubiquitous system in bacteria that is responsible for ferrous iron transport.²² This system is crucial for anaerobic bacteria, playing a role in multiple cellular mechanisms, stress response, and also contributing to virulence.²³ Although both of these genes exhibit only a small increase in expression, the fact that there are multiple genes in this pathway upregulated makes this observation more relevant. It is possible that the observed enhancement of growth at 12 hours, where growth was over 100% of control for all concentrations, is related to this increase in iron uptake. If this is the case, it would indicate that the gene expression pathways activated by *R. gawvreauii* in response to naringenin not only counteract any negative effects of the polyphenol but also are ultimately beneficial to the bacteria itself.

There were only a few genes identified that were downregulated by *R. gawvreauii* in response to naringenin (Table 2). Similar to the profile of upregulation, the gene with the highest

decrease coded for a hypothetical protein. The gene with the second largest decrease in expression was identified as coding for a nucleotidyltransferase, which is a type of enzyme involved in DNA synthesis and repair (Table 2).²⁴ The other genes identified are involved in pathways such as molecular translocation and translation (Table 2). However, none of the genes downregulated by *R. gawvreauii* in response to naringenin exhibited a greater than 1.5-fold change in gene expression. This makes it difficult to say whether the observed change is meaningful or if the difference in expression is simply due to variation.

Ultimately, *R. gawvreauii* was able to maintain a normal phenotype under all concentrations of naringenin tested (Figure 1A and B). Although it was hypothesized that naringenin may affect gene expression of *R. gawvreauii*, it appears that this effect is minimal (Table 1). From these data, it seems logical to conclude that naringenin is unable to affect *R. gawvreauii* to an extent that can be observed through phenotypic or genotypic evaluation.

The addition of naringenin to *B. catenulatum* resulted in 38 genes with a greater than 1.5-fold increase in expression (Table 3). There was an observed upregulation of multiple genes involved in protein synthesis and protein secretion, which is demonstrated by a 2-fold increase in the putative lipoprotein signal peptidase and a 1.6-fold increase in the tryptophan synthase α -subunit gene (Table 3). The lipoprotein signal peptidase is responsible for removing the signal peptide prior to secretion.²⁵ Upregulation of this gene indicates that *B. catenulatum* is producing and secreting more proteins, which may be related to the documented enhancement of growth for all concentrations of naringenin at 12 hours post inoculation.

In response to naringenin, *B. catenulatum* also upregulated multiple genes associated with cell transport. This is demonstrated by a 1.5-fold increase in the dipeptide ABC transporter permease component, the galactoside transport protein, and the copper-transporting ATPase (adenosine triphosphatase) (Table 3). The ABC transporter system is ubiquitous in bacteria and transports small molecules into and out of the cell.^{26,27} The copper-transporting ATPase is an ATP-dependent, transmembrane protein that traffics copper into and out of the cells.²⁸ Copper is essential for multiple metabolic pathways, including detoxification of free radicals.²⁸ An increase in the copper-transporting ATPase would require additional ATP, which may explain the upregulation of 2 genes involved in ATP synthesis (Table 3). It is possible that the increase in cellular transport is a way for *B. catenulatum* to maintain homeostasis in the presence of naringenin.

There were 17 genes identified that had a greater than 2.5-fold decrease in expression for *B. catenulatum* in response to naringenin treatment (Table 4). The largest decrease was observed in the deoxyuridine 5'-triphosphate nucleotidohydrolase gene, with a 3.8-fold change in expression. The deoxyuridine 5'-triphosphate nucleotidohydrolase, or dUTPase, is an enzyme responsible for converting dUTP to dUMP, which is

Table 3. Genes upregulated by *Bifidobacterium catenulatum* in response to naringenin.

GENE ID	GENE DESCRIPTION	FOLD↑	FUNCTION
BBCT_0807	Hypothetical protein	3.7	Unknown
BBCT_146	Conserved hypothetical protein	2.8	Unknown
BBCT_0952	Conserved hypothetical protein	2.2	Unknown
BBCT_0976	Conserved hypothetical protein	2.1	Unknown
BBCT_1237	Putative lipoprotein signal peptidase	2.0	Protein secretion
BBCT_0743	Conserved hypothetical protein	2.0	Unknown
BBCT_0923	Conserved hypothetical protein	2.0	Unknown
BBCT_1231	Conserved hypothetical protein	1.9	Unknown
BBCT_0715	Hypothetical protein	1.9	Unknown
BBCT_1471	ATP synthase epsilon subunit	1.8	ATP synthesis
BBCT_0629	Truncated conserved hypothetical protein	1.8	Unknown
BBCT_0233	Conserved hypothetical protein	1.7	Unknown
BBCT_0204	Phosphopantetheine adenylyltransferase	1.7	Metabolism
BBCT_0970	Holliday junction DNA helicase RuvA	1.7	DNA repair
BBCT_0948	Conserved hypothetical protein	1.7	Unknown
BBCT_1598	Truncated conserved hypothetical protein	1.6	Unknown
BBCT_1557	Conserved hypothetical protein	1.6	Unknown
BBCT_1216	Conserved hypothetical protein	1.6	Unknown
BBCT_0983	Tryptophan synthase alpha subunit	1.6	Protein synthesis
BBCT_0680	Transcriptional regulator	1.6	Transcription
BBCT_1093	Putative acetyltransferase	1.5	Metabolism
BBCT_1624	Conserved hypothetical protein	1.5	Unknown
BBCT_1013	Hypothetical protein	1.5	Unknown
BBCT_1342	Copper-transporting ATPase	1.5	Copper transport
BBCT_1688	Conserved hypothetical protein	1.5	Unknown
BBCT_1169	Dipeptide ABC transporter permease component	1.5	Molecular transport
BBCT_1680	Galactoside transport protein	1.5	Molecular transport
BBCT_0013	Conserved hypothetical protein	1.5	Unknown
BBCT_1574	<i>N</i> -acetylglucosamine-6-phosphate deacetylase	1.5	Metabolism
BBCT_0175	6-phosphogluconate dehydrogenase-like protein	1.5	Metabolism
BBCT_0364	Conserved hypothetical protein	1.5	Unknown
BBCT_0688	Conserved hypothetical protein	1.5	Unknown
BBCT_0180	Conserved hypothetical protein	1.5	Unknown
BBCT_1635	Hypothetical protein	1.5	Unknown
BBCT_1478	ATP synthase subunit A	1.5	ATP synthesis
BBCT_0202	Conserved hypothetical protein	1.5	Unknown
BBCT_1379	Conserved hypothetical protein	1.5	Unknown
BBCT_0529	Conserved hypothetical protein	1.5	Unknown

Table 4. Genes downregulated by *Bifidobacterium catenulatum* in response to naringenin.

GENE ID	GENE DESCRIPTION	FOLD ↓	FUNCTION
BBCT_1116	Deoxyuridine 5'-triphosphate nucleotidohydrolase	3.8	Thymidine biosynthesis
BBCT_0098	Transcriptional regulator	3.3	Transcription
BBCT_1543	UDP-galactopyranose mutase	3.3	Cell wall synthesis
BBCT_0990	Ribosome recycling factor	3.2	Protein synthesis
BBCT_1179	Nicotinate-nucleotide pyrophosphorylase	3.1	Metabolism
BBCT_0954	Conserved hypothetical protein	3.1	Unknown
BBCT_1033	Arginine repressor	2.9	Transcription
BBCT_1640	Hypothetical protein	2.8	Unknown
BBCT_1011	Carbohydrate kinase	2.8	Metabolism
BBCT_1078	Conserved hypothetical protein	2.8	Unknown
BBCT_1329	Putative transposase	2.7	Stress response
BBCT_1062	DNA ligase	2.6	DNA repair
BBCT_0095	Putative glutaredoxin	2.6	Stress response
BBCT_1163	Conserved hypothetical protein	2.5	Unknown
BBCT_1455	L-ribulose-5-phosphate 4-epimerase	2.5	Metabolism
BBCT_0034	Putative phosphoprotein phosphatase	2.5	Metabolism
BBCT_0008	Glutamate dehydrogenase	2.5	Metabolism

then used to synthesis thymidine.²⁹ The dUTPases are ubiquitous to all life-forms and without it, uracil is incorporated into DNA, leading to DNA breaks and ultimately cell death.²⁹ Interestingly, there was a 3.3-fold decrease in the uridine diphosphate (UDP)-galactopyranose mutase, an enzyme that converts UDP-Galp to UDP-Galf, which is a main constituent of the bacterial cell wall and surface.³⁰

There are also 5 separate genes downregulated that are involved with metabolism, including a 2.5-fold decrease in expression of L-ribulose-5-phosphate 4-epimerase (Table 4). This epimerase functions to interconvert L-ribulose 5-phosphate and D-xylulose 5-phosphate.^{31,32} This conversion is the final step in L-arbinose production, which is then used as a carbon source.³³ A decrease in genes involved with metabolism would indicate a drop in catabolism and/or energy production. Taken together, these results indicate that in response to naringenin, *B. catenulatum* downregulates genes associated with metabolism, catabolism, and cell growth.

Despite limited changes to the phenotype of *B. catenulatum*, gene expression analysis revealed that the addition of naringenin produced substantial changes in gene regulation. The addition of naringenin resulted in upregulation of protein synthesis and protein secretion, molecular transport, and ATP synthesis (Table 4). However, there was downregulation of multiple genes involved in critical pathways, such as thymidine biosynthesis, cell wall construction, and metabolism (Table 4).

Downregulation of these genes does seem counterintuitive and would indicate a decrease in growth and cell replication. However, this downregulation was not observed in the phenotype. In fact, primary results demonstrated that for all concentrations of naringenin tested, growth of *B. catenulatum* was enhanced at 12 hours, with no difference from the control at 24 hours post inoculation. Therefore, it can only be concluded that the genes downregulated were expendable, at least temporarily, or that they were functionally redundant. In the end, *B. catenulatum* was able to maintain a normal phenotype for all concentrations of naringenin tested. This would indicate that the changes in gene expression were necessary, and provided what was needed to overcome any deleterious effects caused by naringenin.

The addition of naringenin to *E. caccae* resulted in 28 genes with a greater than 1.5-fold increase in expression. However, 19 of these were hypothetical proteins, which unfortunately do not provide any relative information on their function (Table 5). Besides hypothetical proteins, there are 3 genes upregulated that are involved in transcription: 2 transcriptional regulators with a 1.7- and 1.6-fold increase and 1 RNA-binding protein with a 1.6-fold increase (Table 5). This indicates an overall increase in transcription for *E. caccae* in response to naringenin. There is also an observed 1.6-fold increase in the preprotein translocase subunit SecG. SecG is a part of the SecYEG complex, which is located in the membrane and functions to actively

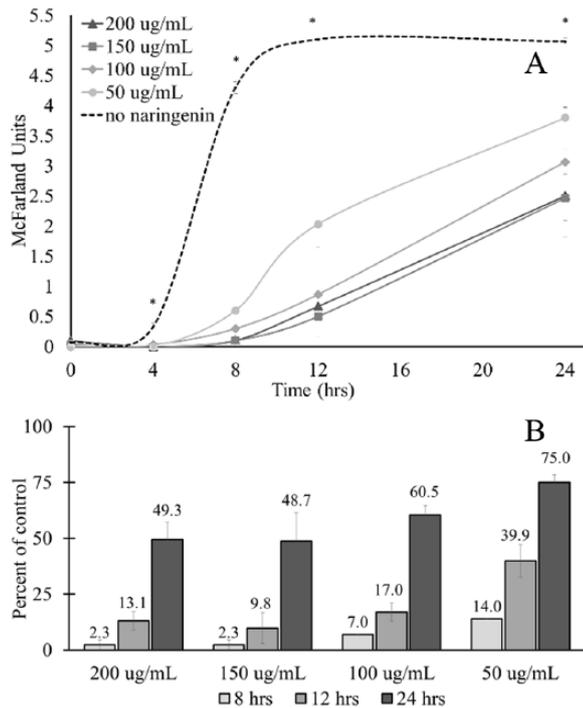


Figure 3. The effect of naringenin on the growth of *Enterococcus caccae*. *Enterococcus caccae* was cultured for 24 hours in the presence of increasing concentrations of naringenin. A densitometer was used to measure the McFarland units at designated time points. The * mark indicates that there was a statistically significant difference between the control and experimental groups at that time point, according to a 2-tailed, Student *t* test ($P < .05$). (A) The 24-hour growth curve of *E caccae* supplemented with increasing concentrations of naringenin. The dotted line represents bacteria grown without naringenin. (B) The percent of control for *E caccae* cultured in each concentration of naringenin at 8, 12, and 24 hours post inoculation.

transport proteins across the membrane.³⁴ This indicates an increase in protein transport and is possibly used as a mechanism for the cell to maintain homeostasis.

There were 49 genes with a greater than 1.5-fold decrease in activity for *E caccae* in response to naringenin. The gene that demonstrates the most decrease, a 2.0-fold change, was identified as a peroxiredoxin gene (Table 6). This gene codes for a thiol-specific antioxidant that functions using peroxidase activity.³⁵ Importantly, there was a decrease in 5 individual genes involved in the phosphotransferase system (PTS) pathway. The PTS β -glucoside transporter subunit EIIBCA, with a 1.9-fold decrease; a PTS mannose/fructose/sorbose family IIA and a IID component, both with a 1.6-fold decrease; a PTS IIC component, with a 1.5-fold decrease; and a PTS trehalose IIBC component, with a 1.5-fold decrease in expression (Table 6). All of these are components of the carbohydrate PTS system and function to bring in sugar and carbohydrates into the cell.³⁶ A decrease in so many of the genes responsible for this pathway would most likely limit the ability for *E caccae* to maintain energy production and normal growth. This is supported by a 1.8-fold decrease in the teichoic acid glycosylation protein, teichoic acid is a component of gram-positive cell membrane,

wall, and capsule,³⁷ a 1.5-fold decrease in the 6-phosphofructokinase, which is part of the glycolysis pathway³⁸ and the downregulation of 3 other genes involved in metabolism (Table 6). Ultimately, the growth of *E caccae* was severely inhibited due to the addition of naringenin. Therefore, it is logical to say that any changes in the pattern of gene expression due to the addition of naringenin were only able to partially counteract any negative effects or were ineffectual.

Conclusions

The results of these experiments clearly show that each bacterial strain tested responded to naringenin differently, both in terms of growth pattern and gene expression. Treatment with naringenin did not measurably affect the growth of *R gaurvreauii*, for *B catenulatum* there was an increase in growth at 12 hours post inoculation, and for *E caccae*, a drastic inhibition of growth for each time point tested. The addition of naringenin was determined to produce changes to the gene expression for all 3 of these strains. *Ruminococcus gaurvreauii* had an increase in genes involved with iron uptake and *B catenulatum* had an upregulation in genes involved in DNA repair and molecular transport and decrease in expression of genes involved in metabolism and thymidine biosynthesis. *Enterococcus caccae* responded to naringenin by upregulating transcription pathways and protein transport, whereas downregulating sugar transport and purine synthesis.

Taken together, this provides valuable information regarding the way that different types of microbes may respond to the same stimuli. In the context of the gut microbiota, there are 500 to 1000 species of bacteria present at any given time.

The data shown here indicate that each species may respond in a different manner, and the interaction between bacteria and naringenin can be considered somewhat dynamic.

R gaurvreauii, *B catenulatum*, and *E caccae* were used in this study as representatives of different strains that compose the gut microbiota. It would be valuable, in future work, to see whether the changes in *R gaurvreauii*, *B catenulatum*, and *E caccae* are representative of the effect that would occur at a genus or class level. It would also be interesting to see whether or not naringenin would have similar effects on a gut microbiota community. It is quite possible that the addition of polyphenols such as naringenin has a direct hand in shaping the community structure of the gut microbiota, by affecting both growth and gene expression of the bacteria present.

Author Contributions

JF, LSL, MW, and WX conceived and designed the experiments. LZ and GAA analyzed the data. JF wrote the first draft of the manuscript. PT, SP, and MK contributed to the writing of the manuscript. JF, LSL, MW, WX, LZ, GAA, PT, SP, and MK agree with manuscript results and conclusions. JF, LSL, MW, WX, and SP jointly developed the structure and arguments for the paper. JF, LSL, MW, WX, LZ, GAA, PT, SP, and

Table 5. Genes upregulated by *Enterococcus caccae* in response to naringenin.

GENE ID	GENE DESCRIPTION	FOLD↑	FUNCTION
UC7_RS13995	Hypothetical protein	2.1	Unknown
UC7_RS15645	Hypothetical protein	2.1	Unknown
UC7_RS13400	Hypothetical protein	2.1	Unknown
UC7_RS13840	Hypothetical protein	1.9	Unknown
UC7_RS13985	Hypothetical protein	1.8	Unknown
UC7_RS13845	Hypothetical protein	1.8	Unknown
UC7_RS14745	Transcriptional regulator	1.7	Transcription
UC7_RS12590	Hypothetical protein	1.7	Unknown
UC7_RS15085	Hypothetical protein	1.7	Unknown
UC7_RS14600	DNA helicase	1.7	DNA replication
UC7_RS14805	ATP-grasp domain-containing protein	1.7	Energy utilization
UC7_RS13975	Hypothetical protein	1.6	Unknown
UC7_RS14295	Preprotein translocase subunit SecG	1.6	Protein translocation
UC7_RS16575	Hypothetical protein	1.6	Unknown
UC7_RS13125	Hypothetical protein	1.6	Unknown
UC7_RS12740	Hypothetical protein	1.6	Unknown
UC7_RS15375	Transcriptional regulator	1.6	Transcription
UC7_RS16265	RNA-binding protein	1.6	Transcription
UC7_RS14730	Nucleotide sugar dehydrogenase	1.6	Metabolism
UC7_RS12975	Hypothetical protein	1.6	Unknown
UC7_RS13675	Hypothetical protein	1.6	Unknown
UC7_RS15295	Hypothetical protein	1.5	Unknown
UC7_RS13470	Hypothetical protein	1.5	Unknown
UC7_RS16155	Hypothetical protein	1.5	Unknown
UC7_RS14625	Hypothetical protein	1.5	Unknown
UC7_RS12585	Cell division protein ZapA	1.5	Cell division
UC7_RS16570	Hypothetical protein	1.5	Unknown
UC7_RS16330	Acyl carrier protein	1.5	Protein synthesis

Table 6. Genes downregulated by *Enterococcus caccae* in response to naringenin.

GENE ID	GENE DESCRIPTION	FOLD↓	FUNCTION
UC7_RS15835	Peroxiredoxin	2.0	Stress response
UC7_RS13735	Hypothetical protein	2.0	Unknown
UC7_RS13805	Hypothetical protein	1.9	Unknown
UC7_RS13955	Protein-(glutamine-N5) methyltransferase, release factor-specific	1.9	Methylation
UC7_RS14195	PTS β -glucoside transporter subunit EIIBCA	1.9	Sugar transport
UC7_RS15880	Hypothetical protein	1.9	Unknown

Table 6. (Continued)

GENE ID	GENE DESCRIPTION	FOLD↓	FUNCTION
UC7_RS14150	Serine hydrolase	1.8	Metabolism
UC7_RS16020	Hypothetical protein	1.8	Unknown
UC7_RS14330	Teichoic acid glycosylation protein	1.8	Cell wall synthesis
UC7_RS14840	Hypothetical protein	1.8	Unknown
UC7_RS13720	Hypothetical protein	1.8	Unknown
UC7_RS11285	Hypothetical protein	1.8	Unknown
UC7_RS13055	Amidophosphoribosyltransferase	1.8	Purine synthesis
UC7_RS11075	Holliday junction resolvase RecU	1.7	DNA repair
UC7_RS16300	β -hydroxyacyl-ACP dehydratase	1.7	Protein synthesis
UC7_RS11225	Hypothetical protein	1.7	Unknown
UC7_RS12935	Phosphopantetheine adenylyltransferase	1.7	Metabolism
UC7_RS11045	Hypothetical protein	1.7	Unknown
UC7_RS14715	Hypothetical protein	1.7	Unknown
UC7_RS15840	Hypothetical protein	1.7	Unknown
UC7_RS15355	Hypothetical protein	1.7	Unknown
UC7_RS13565	Hypothetical protein	1.7	Unknown
UC7_RS14510	PTS mannose/fructose/sorbose family, IIA component	1.6	Sugar transport
UC7_RS11425	Histidinol-phosphate aminotransferase	1.6	metabolism
UC7_RS15565	PTS mannose/fructose/sorbose family, IID component	1.6	Sugar transport
UC7_RS10845	Hypothetical protein	1.6	Unknown
UC7_RS15915	Hypothetical protein	1.6	Unknown
UC7_RS12815	Hypothetical protein	1.6	Unknown
UC7_RS15525	Ureidoglycolate dehydrogenase	1.6	Purine metabolism
UC7_RS11535	Hypothetical protein	1.6	Unknown
UC7_RS11490	FMN reductase	1.6	Metabolism
UC7_RS14490	Hypothetical protein	1.6	Unknown
UC7_RS11520	Hypothetical protein	1.6	Unknown
UC7_RS10980	Hypothetical protein	1.6	Unknown
UC7_RS14385	Hypothetical protein	1.6	Unknown
UC7_RS15235	Hypothetical protein	1.6	Unknown
UC7_RS13770	CAAX amino protease	1.5	Protein synthesis
UC7_RS15515	Hypothetical protein	1.5	Unknown
UC7_RS14435	Hypothetical protein	1.5	Unknown
UC7_RS12990	6-phosphofructokinase	1.5	Glycolysis
UC7_RS15570	PTS IIC component	1.5	Sugar transport
UC7_RS12210	DNA-directed RNA polymerase subunit delta	1.5	Transcription
UC7_RS13285	PTS trehalose IIBC component	1.5	Sugar transport

(Continued)

Table 6. (Continued)

GENE ID	GENE DESCRIPTION	FOLD↓	FUNCTION
UC7_RS15260	Hypothetical protein	1.5	Unknown
UC7_RS14935	Ribosomal RNA large subunit methyltransferase A	1.5	Protein synthesis
UC7_RS14065	Hypothetical protein	1.5	Unknown
UC7_RS15925	ABC transporter ATP-binding protein	1.5	Molecular transport
UC7_RS14080	ModE molybdate transport repressor domain-containing protein	1.5	Molecular transport
UC7_RS13105	Xanthine phosphoribosyltransferase	1.5	Purine metabolism

MK made critical revisions and approved final version. All authors reviewed and approved the final manuscript.

Disclosures and Ethics

As a requirement of publication, authors have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality, and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. The external blind peer reviewers report no conflicts of interest.

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