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Effects of Broccoli and Carrots on Fecal MicroRNA Expression in Infants:
A Short-term Feeding Study

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Human Environmental Sciences

by

Kaleigh E. Beane
University of Arkansas
Bachelor of Science in Human Nutrition and Hospitality Innovation, 2018

July 2020
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

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Abstract

Diets, through multiple mechanisms, cause significant impact on human health and disease etiology; proposed modes of action include modulation of non-coding RNAs and influences on the gut microbiome. Specifically, phytochemicals change human miRNA expression thereby impacting gut microbiome and/or increasing host immune functions. Apiaceous and cruciferous vegetables have been linked to decreased chronic inflammation, which is closely related with the host immune system, yet it is not investigated how these two classes of vegetables influence the colonic miRNA profile. In this study, therefore we aimed to determine the effects of short-term feeding of broccoli and carrot to infants on fecal miRNA. Three experimental groups were included: (1) control group (CON); (2) carrot group (CRT); and (3) broccoli group (BROC). Participants began the five-day study at six months of age. The puree diets (either broccoli or carrot puree) were introduced from Day 1 through Day 3 (i.e., 3 consecutive days: 2 oz per day). During the intervention period (Day 1 - Day 3), the CON group participants continued consuming breast milk and/or formula only. Diapers with stool were collected on Day 0, Day 2, and Day 4. Fecal RNA was isolated and then miRNA expression was profiled using the NanoString nCounter platform. Additionally, we subjected the miRNA results to Ingenuity Pathway Analysis (IPA) and Gene Ontology Protein Analysis Through Evolutionary Relationships (PANTHER) to predict enriched biological functions/molecular functions in broccoli- and carrot-fed infants. Compared to the CON group, 22 miRNAs and 20 miRNAs passed our criteria to be considered 'differentially expressed' from the CRT and BROC intervention groups, respectively. In our miRNA target prediction analyses, using IPA, a total of 132 potential mRNA target transcripts were identified in the CRT group. Similarly, in the BROC group, a total of 101 potential mRNA target transcripts was predicted. In the subsequent

PANTHER analyses, ‘Positive regulation of cellular response to macrophage colony-stimulating factor stimulus’ and ‘Positive regulation of response to macrophage colony-stimulating factor’; ‘Interleukin-1, type I, activating receptor activity’; ‘Interleukin-1 receptor activity’; and ‘Interleukin-1 receptor binding’ were found to be most significantly enriched in the CRT infants. On the other hand, in the BROC group, ‘Negative regulation of natural killer cell differentiation involved in immune response’; ‘Regulation of natural killer cell differentiation’; ‘Interleukin-1, type I, activating receptor activity’; ‘Interleukin-1 receptor activity’; and ‘Interleukin-1 receptor binding’ were enriched the most. Overall, our study provides evidence that CRT and BROC intervention impacts human infants’ miRNAs and may lead to overall benefits in their immune system likely through different miRNA, predicted mRNA target transcripts, and molecular/biological functions.

Acknowledgements

There are multiple individuals I would like to acknowledge, whom aided in my thesis process and encouraged me to succeed. First and foremost, I would like to show my upmost appreciation for my advisor, Dr. Jae Kyeom Kim. He was always available for any question, guidance, or problem at hand to offer advise as I joined the world of research. He continually displayed a character of kindness, dedication, and encouragement as he challenged me to explore all opportunities in research. Without whom, I am sure my thesis would not have been completed. I would also like to thank Dr. Jeong Hoon Pan, our Post Doctorate. Without his guidance and training my laboratory techniques would be minimum at best. I would also like to thank my entire laboratory team, Mersady Redding, Jingsing Tang, Dr. Jeong Hoon Pan, and Dr. Jae Kyeom Kim, for the constant encouragement, positive attitudes, laughs and memories over the past years. You all made my experience memorable and enjoyable, and I am truly thankful.

Additionally, I would like to thank my committee member Dr. Sabrina P. Trudo, for her constant guidance and dedication as my academic advisor, and for her guidance in human intervention trail methodology. I am also grateful for my committee co-chair, Dr. Betsy Garrison, who was dedicated and pivotal in an easy transition between years, allowing my primary focus to remain of my research. Also thank you to my committee member Dr. Jiangchao Zhao, for broadening my knowledge and understanding of the human gut microbiome.

I would like to send a thank you to more colleagues and friends, my nutrition mentor Mechelle Bailey, my boyfriend Wesley France, Sarah Ann Pendergraft, Natalie Miller, Alexa McLain, Eric McLain, and Lauren Meeker. I owe a great deal of my success to their encouragement and friendship and will cherish the memories of graduate school as I continue in my education.

Lastly, a large amount of gratitude and thanks to my loving parents. Their constant love, support, and faith in this process allowed me to remain motivated and confident. I am genuinely so appreciative of all that they do. They not only support me to accomplish my dreams, but also encourage me to dream bigger once I achieve them.

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Chapter 1

Review of Literature

What is MicroRNA?

Diet elicits significant impacts on human health and disease etiology with numerous mechanisms thus far proposed; modulation of non-coding RNAs is one of them [(1)]. MicroRNAs (miRNAs) are one class of small non-coding RNAs (ncRNAs) and are mostly 20-22 nucleotides in length. MiRNAs are the most abundant class of non-coding RNAs; these small RNAs inhibit gene expression at the post-transcriptional level [(2)]. Specifically, miRNAs are able to repress the translation process of mRNA into proteins via perfect and/or imperfect base-pairing binding thereby inducing mRNA degradation. As it is estimated that approximately one third of all human genes is impacted by this class of small RNAs, it is not surprising that miRNAs play pivotal roles in key biological processes; these include development, differentiation, cell death, and proliferation [(3)].

Biogenesis of MicroRNAs

Small ncRNAs can be divided into a few sub-classes: short interfering RNAs, miRNAs, Piwi-interacting RNAs, repeat-associated small interfering RNAs, natural antisense transcript-derived siRNA, trans-acting siRNA, and tiny ncRNAs [(4)]. MiRNAs are the largest class in human tissues, and are produced by a collective machinery of RNase III proteins [i.e., Drosha, and Dicer [(5)]]. In humans, most miRNAs are encoded in intron regions, while some are present in exon regions [(6)]. Precise miRNA promoters for most miRNAs have been identified through collective analyses of CpG islands and RNA sequencing data [(7)]. MiRNAs often have multiple transcription start sites [(7)], and may occasionally have distinct promoters from their host genes [(8)].

Primary transcripts (also known as pri-RNA) are generated by RNA polymerase II, resulting in short hairpin RNAs (pre-miRNAs) which will form secondary structures to yield mature miRNAs [9; 10]. After transcription, pre-RNAs undergo maturation steps to result in production of miRNAs via the canonical pathway. Processing in the nucleus of pre-miRNAs is mediated by an RNase III protein (Drosha), to predetermine the mature miRNA sequence via specific RNA cleavage [11; 12]. Once translocated to the cytoplasm, other cytoplasmic RNase III proteins (i.e., Dicer) act on the pre-RNA to release a mature double-stranded miRNA [13; 14]. Post Dicer processing, double-stranded miRNAs are unwound and "activated" via the incorporation into RNA-induced silencing complex (RISC). Association with RISC allows for the silencing capability of miRNA on target mRNA [15].

In addition to the canonical pathway of miRNA biogenesis, there is a non-canonical pathway; which occurs when cells are deficient in DiGeorge syndrome critical region 8 (DGCRB), Drosha or Dicer, where miRNA can be produced in a microprocessor-independent manner [16; 17]. The non-canonical pathway starts with the bypass of the Drosha-mediated processing step, and the pre-RNA is generated through mRNA splicing; this process bypasses the transcription-generated short hairpin RNAs [18]. After the bypass, the pre-miRNA continues in the latter steps of the canonical pathway [19]. However, most miRNAs are produced by the canonical pathway, and those produced by the non-canonical pathway tend to be low in number [5].

Control Points of MicroRNAs

There are multiple control points of miRNA biogenesis to fine-tune their expression and to control biological functions. Although not fully understood, several regulatory factors have been proposed thus far. The first possible regulatory mechanism includes Drosha binding proteins,

which either positively or negatively modulate Drosha-mediated processes [20; 21]. Another possible control point relates to Drosha/DGCRB expression, which has been shown to alter expression levels of miRNA [22; 23]. Expression of exportin-5 could also modulate levels of miRNAs; the exportin-5 is responsible for transport of miRNAs from nucleus to cytoplasm [24]. In the cytoplasm, Dicer binding proteins [i.e., TAR RNA binding protein and protein kinase RNA activator] may regulate miRNAs through impacting the RISC assembly [25; 26]. Additionally, studies suggest that epigenetic controls contribute to miRNA regulation as well [27]. Specifically, miRNAs may be regulated through epigenetic control of multi-drug resistance-1, where miRNAs could be directly repressed [28]. Lastly, genetic single nucleotide morphisms (SNPs) within the miRNA may alter the processing efficiency [29].

Although there is convincing evidence showing that miRNA play a role in post-transcriptional regulations, specific mechanisms by which miRNAs control mRNA expression is still not fully understood. One possible mechanism is that miRNAs may inhibit mRNA initiation by interfering with the recognition of M7G-cap [30]. Additionally, promotion to drop-off ribosomes by miRNA was a proposed mechanism of mRNA repression [31]. Another proposed mechanism is through inhibition of actively translating polyribosomes [32]. Lastly, miRNA-induced deadenylation may possibly result in mRNA decay in P-body, high levels of which are found in the components of mRNA decay pathway [33].

In the following sections we discuss evidence of the potential for dietary elements, and their constituents, to regulate miRNA, and thereby influence target mRNA expression. As specific mechanisms between miRNA and mRNA remain relatively unknown, the mechanistic influence of diet remains unclear as well. Little is known of how miRNAs are impacted by diet, and of potential targeted regulatory mechanisms of dietary constituents. Further research is needed to

identify and solidify the mechanisms of miRNA control on mRNA, as well as the target mechanisms impacted via dietary interventions.

Effects of Vegetables and Their Bioactives on MicroRNA

Phytochemicals are a diverse group of plant-derived bioactive compounds, including but not limited to: polyphenols (i.e., flavonoids), carotenoids, glucosinolates, phytosterols, and allicins [reviewed in [34]]. In addition, plant secondary metabolites (e.g., polyphenols) are phytochemicals found in many plant foods, and are the most abundant source of antioxidants within the diet [35; 36]. Dietary sources of each phytochemical category are included in Table 1s.

Accumulating evidence shows that dietary constituents influence miRNA expression, thereby resulting in an array of downstream mRNA expression changes. Specifically, polyphenols in fruits and vegetables alter miRNA expression. Phenolic constituents from mango were found to modulate inflammation by regulating the expression of miR-126. Kim et al. demonstrated that mango bioactives increased miR-126 expression which in turn suppressed the phosphatidylinositol-3-kinase (PI3K)/mammalian target of rapamycin (mTOR)/protein kinase B (AKT) signaling axis [37]. Another study showed that pomegranate ellagitannins and anthocyanins upregulated miR-126 expression and suppressed PI3K signaling, thereby reducing mucosal inflammation in carcinogen-treated rats [38]. Last, blueberries, rich in polyphenols, showed an inhibition of atherosclerosis-related miRNAs (i.e., miRNAs miR-21, miR-146a, and miR-125b) when looking at the effect of polyphenol fractions in inflammation among RAW 264.7 cells [39]. In the context of inflammation, polyphenols from green tea (i.e., catechins) modulate the miRNA profile in white adipose tissue in mice fed a high-fat diet. Specifically, in adipose tissue, miR-335 is known to be upregulated by TNF-α, a proinflammatory cytokine[40].

However, the TNF-α-mediated upregulation of miR-335 in adipocytes was inhibited through catechins in green tea extract, and thereby reduced mRNA expression associated with insulin resistance and lipid metabolism (e.g., Insr, Irs1, Sirt1, and Foxo1). Thus, it is indicated that obesity leads to a pro-inflammatory state, and gene expression profiles associated with insulin resistance are counteracted by green tea extract catechins via miRNA regulation and increased lipolysis [(40)].

In addition to polyphenols, in a recent study, Slaby et al. found that three miRNAs (miR-155, miR-23b, and miR-27b) were affected by iberin and sulforaphane (from the isothiocyanate class of dietary bioactives) in colon epithelial cell lines (NCM460 and NCM356)[(41)]. This study showed possible roles of miRNAs in chemoprevention through targeting multiple genes harboring SNPs (e.g., AGTR1, TNFAIP2, and SIRT5). Interestingly, DICER1, a gene involved in the generation of miRNAs, was predicted as a target of miR-23b and was significantly associated with decreased risk of sporadic colorectal cancer [(41)]. Similarly, in a recent study, butyrate, a metabolite of dietary fibers, was shown to regulate miRNAs; there was a reduction of miR-106b, which may be involved in inhibition of LT97 colon adenoma cell proliferation[(42)]. The modification of miR-106b expression might contribute to the induction of cell cycle regulatory proteins, like p21, as a mechanism of chemoprevention [(42)]. These studies reinforce the idea that dietary bioactives may not only influence colonic miRNA expression through their direct modulation, yet also influence secondary metabolites produced by gut microbiome (e.g., butyrate).

Gut Microbiome and Health

The human gut microbiome consists of over 100 trillion bacteria, fungi and viruses. This plethora of microbes contributes more than 150 times more genetic information to the host than the human genome itself [(43)], playing a key role in host health and diseases through this genetic information. Advances in technology have improved the exploration of the human microbiome, allowing for the categorization of the gut microbiome per operational taxonomic (OTUs) units based on bacterial 16S ribosomal DNA. Now, vast studies are generating a map of the entire metagenome to better understand functional signatures of the gut microbiome, and assess one's health status [i.e., Crohn's disease [(44)]]. Diet is one of multiple factors contributing to the functional signatures of the gut microbiome. For instance, studies show long-term, high-fiber dietary exposure influences the gut microbiome in healthy young participants [(45)] and elderly participants [(46)], supporting the notion of diet as an important contributor to the health of the host's gut microbiome.

Types of fibers and dietary sources

Dietary fibers have two subcategories, non-fermentable/insoluble and fermentable/soluble forms, which have varying impacts on the health of the host. The division of fibers is based off of differing physiochemical characteristics [e.g., origin, solubility and viscosity, fermentability, and chemical structure [(47)]]. Both soluble and insoluble dietary fibers have beneficial physiological effects on the host [e.g., body weight management [(48)]].

Soluble fibers (e.g., pectin, guar gum, and some inulin) are present in whole grains, legumes, seeds and nuts, and some fruits and vegetables (e.g., carrots, apples) [(49)]. One of the defining physiochemical characteristics of soluble fibers includes viscosity, which slows absorption of

nutrients in the intestine [e.g., glucose and lipids [47]] by creating a gel-like form. The capability of soluble fiber (e.g., pectin) to slow the absorption of glucose has been shown to aid preventative measures against metabolic diseases by controlling blood glucose levels [50].

Insoluble dietary fibers [e.g., celluloses, hemicellulose, and fructans [51]] are present in nuts, beans, some whole grains (e.g., whole wheat flour and brown rice), and vegetables [52; 53]. The defining physiochemical characteristics of insoluble fiber include a bulking effect and fermentation by the gut microbiome. Insoluble dietary fiber fermentation by the host gut microbiome produces short-chain fatty acids (SCFAs) as secondary by-products, aiding in a healthy microbial composition within the host gut microbiome [47]. SCFAs are critical to the health of the host due to their potential immunological influences [e.g., butyrate's capability to increase expression of antibacterial and host defense genes [54]]. A fiber-deprived gut microbiome has shown to increase disease susceptibility due to a reduction in protective mucus, which is utilized as a defense layer to block pathogens from entering the system [55], confirming the beneficial impact of fiber on the health of the host.

Prebiotics were originally defined as "non-digestible compounds that, when consumed, induce changes in composition and/or activity of the gastrointestinal bacteria, thus causing benefit(s) upon host health" [56]. Prebiotics include, but are not limited to, insoluble fibers. Consumption of prebiotics (e.g., β-glucan, xylooligosaccharides, and pure inulin) has shown to influence gut microbiota diversity and to increase production of SCFAs [52; 57; 58; 59]. Positive functional characteristics of prebiotics have been reported including, but not limited to: (a) selective fermentation, (b) modulation of gut pH, (c) fecal bulking, (d) the prevention of gut colonization by pathogens, and (e) the control of putrefactive bacteria, thus reducing the host's exposure to toxic metabolites [60].

Production of SCFAs

SCFAs are produced by the gut microbiota through the fermentation of carbohydrates [50; 52] and other non-absorbable nutrients [57; 59]. The most abundant SCFAs include acetate, propionate, and butyrate [61; 62], and represent 90-95% of all SCFAs produced in the colon [50; 58; 59]. SCFAs are utilized by downstream bacterial species (cross feeding) and/or directly by the host as nutrient sources. Specifically, butyrate was frequently reported to be involved in other functions such as immune regulation [63], cell growth [64; 65], intestinal barrier function [66], and ion transport [67].

Effects of dietary fibers on epithelial barrier functions

The human intestine is a tubular organ, with the outer-most layer made of muscular tissue and the inner-most layer consisting of a single layer of enterocytes interfacing between the host inner tissue and the exterior intestinal environment. The single layered enterocytes are concatenated through tight junctions (TJs) to form a defense for food-borne pathogens or other ingested toxic compounds [68]. The construction of TJs is maintained by enterocytes' histological development and signaling pathways, such as tumor necrosis factor-α [69]. Without this maintenance, TJs are the attack point for pathogenic bacteria to invade the host, resulting in permeability and leading to destructive intravenous electrolyte exchange, microbial dysbiosis, and diarrhea [69].
Dietary fibers influence epithelial barrier functions through the production of SCFAs, specifically butyrate. Butyrate has a beneficial effect on epithelial barrier function, and is known to even nourish TJs (e.g., beta defensin, cingulin, ZO-1 and ZO-2 proteins) in chicken jejunal and cecal explants [70]. It is suggested that butyrate enhances the TJs through activating Akt/mTOR pathways and ATP replenishment [71]. Another potential function of dietary butyrate

includes the repression of interleukin 10 (IL-10) receptor-dependent claudin-2, thereby promoting barrier function by repressing the leak-flux mechanism responsible for diarrhea [72].

Anti-inflammatory effects of dietary fibers

The gut functions as one of the largest immune systems in humans, with physical (i.e., barrier function) and biochemical defenses [73]. Once triggered, the pro-inflammatory response is the primary reaction to pathogens [74]. Cells are then differentiated into multiple sub-types of immune cells as a natural response [75]. These epithelial cells are constantly encountering pathogens and may frequently activate the immune response, resulting in immunologic diseases [e.g., inflammatory bowel disease (IBD) and autoimmune diseases [76; 77]]. Anti-inflammation (i.e., inhibitor of excessive inflammation) is needed to maintain immune homeostasis. Butyrate is known to act as an anti-inflammatory through its modulation of cytokine production, kinase activity, and immune-associated signaling pathways. Butyrate has been linked to multiple anti-inflammatory mechanisms, including the up-regulation of immunosuppressive IL-10 [78], nuclear factor (NF)-κB [79], and histone deacetylase [80; 81]. Thus, supplementation of butyrate dense dietary fiber may counteract the severity of IBD and other inflammatory syndromes [79; 82; 83].

Phytochemicals and Gut Microbiome

The term 'phytochemical' broadly encompasses all plant-derived chemicals. However, the term 'phytochemical' typically is used to refer to small non-essential nutrients with health-promoting actions [84]. About 90%-95% of phytochemicals reach the colon, and are transformed by the resident colonic microbiota [85], yielding bioavailable metabolites. The absorption of phytochemicals have proven beneficial to the health of the host, as being cardioprotective [86; 87],

and protective against glucose toxicity [88]. Additionally, there has been a link with an increase in phytochemicals (i.e., polyphenols) and prevention in metabolic syndrome [89; 90], as well as a potential link to phytochemicals (i.e., hesperidin) and beneficial effects on colorectal cancer [91]. Last, unabsorbed dietary phytochemicals can directly modulate the microbiota. For instance, phenolic compounds from tea leaves inhibited growth and adhesion of *Clostridium Spp*., *E. coli,* and *S. Typhumurium* [92].

Carotenoids and their impacts on gut microbiome

Carotenoids have been linked to a decreased risk of chronic diseases [93], and to the health of the gut microbiome. The effect of carotenoids, commonly found in apiaceous vegetables, on the gut microbiome has been examined previously in two studies. Ramos et al. analyzed the carotenoid source of Tucumã oil and its effect on microbial diversity and SCFA production in cows. Results indicated the use of Tucumã oil as a carotenoid source favorably shifted the gut microbiome. [94]. Comparatively, a long-term randomized control study in humans analyzed the relationship between colonic mucosal bacteria and serum carotenoid concentrations. A shift in 11 operational taxonomic units were associated with higher serum carotenoid levels, as well as other factors (e.g., body mass index, smoking, diet). Thus, indicating both behavioral and metabolic factors may impact the bioavailability of carotenoids to the gut microbiome [93].

Glucosinolates and their impacts on gut microbiome

The effect of glucosinolates, sulfur-containing dietary compounds, on the gut microbiome has only been examined previously in one study. This gap in literature is surprising, as their benefits benefits against chronic disease, such as cancers, are well known [95; 96; 97]. In this study,

Kaczmarek et al. found β-diversity alterations, indicating that bacterial communities were impacted by broccoli feeding[98]. Additionally, broccoli consumption significantly altered a few key metabolic pathways: endocrine system, transport and catabolism, and energy metabolism [98]. Clearly, further studies are warranted in the context of the disease preventive potential of cruciferous vegetables through gut microbiome modulation.

Cruciferous Vegetables and Immune Functions

The consumption of cruciferous vegetables has many health benefits, as they are a good source of glucosinolates and the SCFA butyrate when digested by gastrointestinal microbiota (e.g., *Eubacterium rectale*). In a recent study looking at fiber fermentation by gastrointestinal microbiota, vegetables within the cruciferous family (i.e., broccoli and cauliflower) ranked among the highest in total dietary fiber, substrate disappearance during fermentation, and SCFA production compared to other vegetables (i.e., carrot, celery, onion, radish, cucumber, and lettuce). Specifically, broccoli and cauliflower provided a good source of butyrate, a SCFA[99]. Butyrate plays an essential role in immune defense via the maintenance of the epithelial barrier function, as previously noted. This beneficial effect on the intestinal epithelial barrier has been linked to therapeutic and preventive effects against a number of diseases [i.e., cardiovascular disease [100], hypertension [101], inflammatory disorders [102; 103; 104], and metabolic diseases [105]]. These beneficial effects include, but are not limited to, maintaining TJs within the epithelial wall [106], and mediating immune homeostasis via epithelial biosynthesis of retinoic acid [107]. Thus, cruciferous vegetables (i.e., broccoli) continues to suggest an essential role in immune functions of an individual through the production of the SCFA butyrate via gastrointestinal microbiota digestion.

Additionally, cruciferous vegetables provide the main source of the glucosinolate class of phytochemicals in the diet [108]. Glucosinolates can be hydrolyzed into various secondary metabolites (i.e., isothiocyanates) [109]. With the link between cruciferous vegetables secondary metabolites and therapeutic/preventive effects on a multitude of diseases, the inclusion of these vegetables is vital in dietary intervention of the gut microbiome. More research on the impact of broccoli on the gut microbiome via secondary metabolite production (e.g., isothiocyanates and SCFA) is needed, as only one article has surfaced when searched. Specifically, the impact of broccoli's role in immune function via gut microbiome modulation is relatively unknown, which is somewhat unexpected given its frequent consumption. However, the impact of the SCFA butyrate on immune function via the maintenance of the epithelial barrier has been well established in literature.

Apiaceous Vegetables and Immune Functions

Currently there is a gap in the literature regarding apiaceous vegetables and immune function, however some evidence exists for a role in reduction of inflammation. Apiaceous vegetable dietary constituents, such as falcarinol (FaOH) and falcarindiol (FaDOH, play a primary role in this reduction of inflammation. In a recent study, Kobaek-Larsen et al. reported anti-inflammatory potential of apiaceous vegetables; it was found that FaOH and FaDOH inhibit NF-κB signaling and its downstream inflammatory markers (e.g., COX-2) [110]. Further, it was reported that FaOH reduces inflammation via modulating Nrf2 mRNA [111]. Nrf2 is a transcription factor that upregulates the expression of antioxidant, anti-inflammatory, and cytoprotective enzymes [112]. Additionally, in rats, FaOH and FaDOH modified intestinal microbiota composition by influencing the growth of *Lactobacillus reuteri* and *Turicibacter* [113].

Further investigation is needed regarding the relationship between apiaceous vegetables and inflammatory markers, especially in the context of gut microbial modulation.

Crosstalk Between Gut MicroRNA and Gut Microbiome: Implications in Infants

Studies have showed that the gut microbiota could be regulated by host-secreted miRNAs and, at the same time, the gut microbiota may affect the host by modulating various host miRNAs. To be specific, gut microbes and their metabolites possess a potential to regulate gene expression in intestinal epithelial cells via miRNA modulation and activation of multiple signaling pathways and diverse immune responses. This results in a bidirectional crosstalk between gut miRNA and the gut microbiome [(114)]. In line with this, germ-free antibiotic-treated mice had significantly more fecal miRNA compared to controls with pathogen-free microbiota, indicating that gut microbes contribute to a specific miRNA signature [(115)]. In the study, reduction of luminal miRNAs was associated with a shift in the gut microbiota. It was proposed that host-produced miRNAs are exported into the intestinal lumen as exosomes that are able to drive miRNA to the gut bacteria where they regulate bacteria gene transcription programs [(115)].

As discussed above, clearly the gastrointestinal microbiota is a key factor in optimizing immunity; this is well accepted considering that diverse health benefits (in an array of disease models) can be achieved via modulation of gut microbiome. The foundation of one's gut microbiome is contingent upon 1) acquiring microbiota via mother's milk in infancy and 2) early dietary habits [(116)]. In adults, short-term dietary intervention studies may show change within the microbiota, however maintaining microbial changes in a long-term intervention study has previously been shown to be difficult [(117)]. Therefore, it is vital to establish a healthy gut microbiome as early as possible to ensure maximum health benefits throughout life [(118)]. In the

first years of life (i.e., years 1-3) the microbiota and immune system are not yet matured or established [119], and therefore offer an opportune time to alter the gut microbiome via diet intervention. Thus, infancy proves a critical age group to target food-based intervention in hopes to reduce disease risk and increase immune function. Further, understanding the bidirectional crosstalk between gut miRNA and the gut microbiome would broaden our knowledge as to how the early establishment of the gut microbiome is mediated by host miRNAs, and if so, how diets play roles into mediating host miRNA.

Chapter 2

Methods

Specific Aim and Hypothesis

This study aimed to determine the effects of short-term feeding of broccoli and carrot to infants on fecal miRNA. We hypothesized that broccoli and/or carrot would modulate fecal miRNA expression profiles, thereby potentially targeting key mRNAs involved in innate immunity.

Study Design

The study is a randomized controlled intervention design and each participant provided baseline data prior to their respective vegetable intervention. By the time of recruitment, participating infants were four to five months old thereby they could start the intervention (i.e., semi-solid vegetable food) at the age of six months. The justification for the intervention timing is two-fold: (1) World Health Organization (WHO) recommends starting solid foods at the age of six months [120], and (2) previous studies underscored the importance of the establishment of healthy intestinal microbiota in the first three years of life [119]. The participants were followed for five days.

This study consisted of three intervention groups: (1) control group (CON); (2) carrot group (CRT); and (3) broccoli group (BROC). The CON group participants consumed only breast milk and/or formula, without any vegetables for the intervention period. This would be consistent with the infant's normal diet previously, as we excluded participants who had previous exposure to solid foods. During the intervention period, the CRT group and BROC group consumed carrot puree product and broccoli puree product, respectively, in addition to breast milk and/or formula.

For the vegetable puree, commercially available, frozen, pureed baby food products were purchased from Harvest to Highchair (Charleston, SC, USA). As the product was raw puree, the company did not provide nutritional information on their product's packaging. According to the USDA database, 2 oz of raw broccoli provides 19 kilocalories, 1.5 g of total dietary fiber, 25.85 mg of calcium, 0.4 mg of iron, 175 mg of potassium, 49.05 mg of vitamin C, and 17.05 µg vitamin A. A serving of 2 oz of raw carrots provides 23 kilocalories, 1.6 g of total dietary fiber, 18.75 mg of calcium, 0.17 mg of iron, 182.2 mg of potassium, 3.35 mg of vitamin C, and 475.5 µg vitamin A [121]. Commercial baby food products were chosen for the study, rather than preparing the diet in the lab, thereby providing consistent, well controlled products and enhancing applicability of the study for the general public.

The study lasted a total of five days, starting on the participant's sixth month of age marker. On the first day (Day 0), diapers with fecal matter were collected for characterization of baseline miRNAs. Also, a food recall for the past 10 days (Day -10 through Day 0) was collected; the food recall was provided when subjects (in our study, parents of participating infants) agreed to participate in the study. Through the food recall, information such as amount, types (i.e., breast milk, formula or both), when, and how frequently the participating babies consumed breast milk and/or formula, was recorded. The food recall example is shown in Figure 1.

The puree diets (either broccoli or carrot puree) were introduced from Day 1 through Day 3 (i.e., 3 consecutive days: 2 oz per day). During the intervention period (i.e., Day 1 - Day 3), the CON group participants continued consuming breast milk and/or formula only. The WHO recommends complimentary food at age six to eight months to be offered 1-2 times a day (120). Therefore, our study offered 2 oz of vegetable puree a day; this allows for participating babies to consume the vegetable puree diets 1-2 times per day. All feeding information was collected

using our food recall form. The study design, diaper collection schedule, and dietary intervention information are depicted in Figure 2. This thesis is apart of a larger research project, which extends the intervention, diaper collection, and participants for a total of 30 days. Figure 2 shows the study design for the larger project; however, this thesis stops on Day 4.

Participant Recruitment

Participants were recruited in the Northwest Arkansas region (Washington and Benton counties, AR, USA) for one year (August, 2018- August, 2019). The recruitment was conducted via online news publication (e.g., University of Arkansas News), posted flyers, notification to local daycares, and personal recommendation. Inclusion criteria for participants included: four to five months old at the time of enrollment; willing to avoid over-the-counter and/or prescription medications; willing to exclude certain fruits, vegetables, and herbs from Day -10 through Day 8; willing to comply with the study protocol; and no plan to travel during the study duration. Exclusion criteria included: liver, kidney, or intestinal disorder history; necessary prescription medications; necessary over-the-counter medications; known allergies and/or intolerances to broccoli and carrots; plans to move out of Northwest Arkansas during the study period. If the participants had started solid foods prior to the intervention period, they were excluded from the study. The recruitment was not limited by milk source, breastfed nor formula fed infants. A total of 14 participants were recruited. Two participants dropped out due to occurrence of illness, and one participant failed to comply with the protocol, resulting in a total of 11 participants. The 11 participants were randomly assigned to three groups: CON (n=3), CRT (n=4), and BROC (n=4). Participant demographics (i.e., ethnicity and sex) is provided in Table 2.

Participant Compliance

As aforementioned, participant compliance was monitored using food recalls; each participant received two food recall forms for (1) Day -10 through Day -1; (2) Day 0 through Day 4. The first food recall aimed to gather information as to typical feeding patterns of the infants prior to the study intervention. The second food recall tracked how much of the intervention vegetable was consumed in addition to other diet consumption (e.g., breast milk and/or formula) during the period. In addition, fecal collection questionnaires were provided for asked to record time of diaper collection as well as stool sample conditions (e.g., consistency and color).

Stool Collection

Fecal collection was conducted in the best temperature sensitive way available for the study and participants. Each participant received a cooler with frozen ice packs for diaper collection; participants were instructed to keep the ice packs frozen until the day of the collection where they would put them in the cooler. When a dirty diaper occurred, the participants were instructed to put the diaper in a plastic zip-lock bag and placed in the cooler until the designated collection time. All fecal samples given that day were collected, transferred to the lab, and then stored at -80°C until further analyses.

Fecal RNA Extraction

Fecal RNA from Day 0 and Day 4 was isolated using the RNeasy PowerMicrobiome kit (Qiagen, Hilden, Germany). In short, 0.25 g of stool was placed into a PowerBead tube, and then mixed with 650 µL PM1/βME at maximum vortex speed for 10 minutes. After, the tube was centrifuged at 13,000 × *g* for two minutes and the supernatant was transferred to a clean

collection tube. Next, 150 µL of IRS solution was incubated at 4°C for five minutes and centrifuged at 13,000 × *g* for one minute. Avoiding the pellet, the supernatant was transferred to a clean tube. A solution of 650 µL of PM3 and 650 µL of PM4 was added to each tube. The resulting supernatant was added to a MB RNA Spin Column and centrifuged at 13,000 × *g* for one minute; this step was repeated until all the supernatant had been processed through the spin column. Next, 650 µL of PM5 was added to the MB RNA Spin Column and centrifuged at 13,000 × *g* for one minute. In the center of the column, 50 µL of DNase 1 solution was added and incubated at room temperature for 15 minutes. After, 400 µL of solution PM7 was added and centrifuged at 13,000 × *g* for one minute. Next, 650 µL of solution PM5 was added and centrifuged for one minute. Then, 650 µL of solution PM4 was added and centrifuged. After discarding the flow through, the MB RNA Spin Column was placed into a clean 2 mL collection tube. Lastly, 50 µL of RNase-free water was added to the center of the filter membrane and incubated for one minute. The water was centrifuged at 13,000 × *g* for one minute, and the RNA extraction was completed. The quality of isolated RNA was assessed using the conventional A260/280 ratio and the A260/230 ratio measurement (SpectraMax i3x; Molecular Devices, Sunnyvale, CA, USA). RNA samples were stored in test tubes in -80°C.

Profiling Fecal MiRNAs

Once we confirmed that all extracted RNA samples had a 280/260 ratio of ≥ 1.9 and a 260/230 ratio of ≥ 1.8, extracted RNA was normalized to a single concentration (33 ng/µL) and then loaded into 96-well plates. The 96-well plates were sealed and shipped on dry ice to the University of Minnesota Genomic Center (Minneapolis, MN, USA). Fecal miRNA profiling was performed using the NanoString nCounter platform (NanoString, Seattle, WA, USA). The pre-

built panel for human miRNA (nCounter Human v3 miRNA panel) was used, in which a total of 827 different miRNA were analyzed.

Statistical Analyses and Bioinformatics Analyses

MiRNAs from Day 0 and Day 4 were analyzed using Nanostring nCounter platform to examine impacts of short-term dietary intervention of the vegetables on fecal miRNA expression. First, to ensure that differentially expressed miRNAs were due to dietary intervention and not by other factors, such as infant growth, we normalized the miRNA expression data with their respective baseline which is the data obtained at D0. After, the miRNAs differentially expressed in BROC or CRT were separately compared to the CON by a two-tailed Welch's *t* test. A *p*-value of less than 0.05 was considered statistically significant (Microsoft Excel; Microsoft, Redmond, WA, USA). Data are expressed as mean ± standard error of mean (SEM).

After, for the miRNA dataset, a short-list of differentially expressed genes (DEGs) was generated, using the criteria of miRNA showing a p-value < 0.05 and fold change $> \pm 1.5$. The DEGs were subjected to the IPA software to predict potential mRNA targets, and potentially affected signaling pathways in response to BROC or CRT intervention. After, the predicted mRNA targets were subjected to PANTHER to assess over-represented, or under-represented biological processes and molecular functions in response to BROC or CRT intervention (available at pantherdb.org). We included the top 15 biological processes and molecular functions based on fold enrichment for CRT and BROC.

Chapter 3

Results

Effects of CRT Intervention

Identification of differentially expressed miRNAs in response to CRT intervention

In order to explore impact of CRT intervention on colon miRNA profile, we conducted miRNA expression profiling on infants' fecal samples who were fed two ounces of carrots, per day, over three days. Based on the criteria mentioned above to be considered differentially expressed, a total of 22 miRNAs were included in our DEG list. Of the 22 miRNAs modulated, 14 miRNAs were up-regulated and 8 miRNAs were down-regulated. Those up-regulated by the CRT intervention were miR-1253, miR-130a-3p, miR-146a-5p, miR-147a, miRNA-193-3p, miR-3127-5p, miR-3144-3p, miR-365b-5p, miR-374c-5p, miR-376b-3p, miR-561-5p, miR-595, miR-603, and miR-627-5p. Down-regulated miRNAs by CRT intervention were miR-200c-3p, miR-2116-5p, miR-300, miR-320a-3p, miR-378b, miR-508-5p, miR-521, and miR-885-3p (Table 3).

Computational analysis of miRNA from CRT intervention: prediction of target mRNAs

The twenty-two miRNAs considered differentially expressed were subjected to IPA analysis in order to predict their downstream target mRNAs. Per IPA target prediction, eight miRNAs (miR–130a-3p, miR-146a-5p, miR-147a, miR-193a-3p, miR-200b-3p, miR-320b, miR-378a-3p, and miR-521) collectively targeted 132 mRNAs. It was predicted that some miRNAs targeted only one mRNA (e.g., miR-147a and miR-521) while the others targeted more than one mRNAs (e.g., miR-130a-3p, miR-320a-3p, miR-378b). Of note, it was predicted that miR-146a-5p targets a total of 80 mRNA from CRT intervention, which is the majority of the 132 mRNAs predicted

to be influenced by CRT. miR-193a-3p was predicted to target nine mRNAs [e.g., cyclin D1 (CCND1), E2F transcription factor 6 (E2F6), erb-b2 receptor tyrosine kinase 4 (ERBB4), estrogen receptor 1 (ESR1), MCL1 apoptosis regulator, BCL2 family member (MCL1), plasminogen activator, urokinase (PLAU), protein tyrosine kinase 2 (PTK2), and ribosomal protein S6 kinase, 70kDa, polypeptide 2 (RPS6KB2)] whereas miR-200b targets 23 mRNAs [BRACA1 associated protein 1 (BAP1), engulfment and cell motility 2 (ELMO2), erbb2 interacting protein (ERBIN), ERBB receptor feedback inhibitor 1 (ERRFI1), formin homology 2 domain containing 1 (FHOD1), forkhead box F2 (FOXF2), gem (nuclear organelle0 associated protein 2 (GEMIN2), gse1 coiled-coil protein (GSE1), kelch-like family member 20 (KLHL20), myristoylated alanine rich protein kinase C substrate (MARCKS), phospholipase C gamma (PLCG1), protein phosphatase, Mg2+/Mn2+ dependent 1F (PPM1F), phosphatase and tensin homolog (PTEN), protein tyrosine phosphatase non-receptor type 12 (PTPN12), protein tyrosine phosphatase non-receptor type 13 (PTPN13), protein tyrosine phosphatase receptor type D (PTPRD), arginine-glutamic acid dipeptise (RE) repeats (RERE), WASP family member 3 (WASF3), WD repeat domain 37 (WDR37), zinc finger E-box binding homebox 1 (ZEB1), zinc finger E-box binding homebox 2 (ZEB2), and zinc finger protein, FOG family member 2 (ZFPM2)]. No mRNA targets were identified by IPA for the remaining 14 miRNAs (Table 3).

Gene Ontology prediction of targeted mRNAs in CRT intervention

The PANTHER over-representation tool was used to identify biological processes and molecular functions that were over-represented in the identified target mRNAs. The top 15 identified biological processes and top 15 molecular functions of predicted target mRNAs in CRT intervention group are shown in the Table 5 and Table 6, respectively. Eight over-

represented biological processes had a fold enrichment greater than 100, with $p < 0.001$. Additionally, five over-represented mRNA molecular functions had a fold enrichment greater than 60, with $p < 0.001$. Of note, all of the top three molecular functions significantly over-represented involved interleukin-1 activity, including 'Interleukin-1, type I, activating receptor activity,' 'Interleukin-1 receptor activity,' and 'Interleukin-1 receptor binding.' The targeted mRNA identified in our dataset are associated with 'Interleukin-1, type I, activating receptor activity' included interleukin 1 receptor type 1 (IL1R1) and interleukin 1 receptor like 2 (IL1RL2). The identified target mRNAs, associated with 'Interleukin-1 receptor activity', include interleukin 1 receptor accessory protein (IL1RAP), IL1R1, IL1RL2, and interleukin 1 receptor accessory protein-like 2 (IL1RAPL2) (Table 5). Lastly, molecular function 'Interleukin-1 receptor binding' was associated with seven interleukin mRNAs. MRNAs identified include toll like receptor 9 (TLR9), IL1RAP, interleukin 36 alpha (IL36A), interleukin 1 family member 10 (ILF10), interleukin 36 gamma (IL36G), interleukin 37 (IL37), interleukin 36 receptor antagonist (IL36RN), and interleukin 36 betta (IL36B) as targeted mRNA from our dataset. All biological processes and molecular functions were significantly different in terms of fold enrichment (Table 5 and Table 6).

Effects of Broccoli Intervention

Identification of differentially expressed miRNA in response to BROC intervention

Similar to the CRT intervention, in order to identify global impacts of BROC intervention on colonic miRNAs, we conducted miRNA expression profiling using fecal samples from infants who received two ounces of broccoli, per day, over three days. Based on the criteria aforementioned, a total of 20 miRNAs were included in our list. Of the 20 miRNAs modulated,

nine miRNAs were up-regulated and 11 miRNAs were down-regulated. Those up-regulated by the BROC intervention were: miR-146a-5p, miR-190b, miR-22-3p, miR-3934-5p, miR-409-5p, miR-520f-3p, miR-548i, miR-573, and miR-612. Those down-regulated by BROC intervention were: miR-205-5p, miR-2116-5p, miR-329-3p, miR-361-5p, miR-3613-3p, miR-4425, miR-4443, miR-499a-3p, miR-503-3p, miR-579-5p, and miR-596 (Table 4).

Computational analysis of miRNA from BROC intervention: prediction of target mRNAs

Twenty miRNAs met the screening criteria for IPA analysis to predict target mRNAs. Six miRNAs collectively targeted 101 mRNAs from our dataset. MiR-361-5p only targeted one mRNA while others were predicted to interact with multiple transcripts (e.g., miR-22-3p targeting five mRNAs [bone morphogenetic protein 7 (BMP7), ESR1, MYC associated factor (MAX), peroxisome proliferator activated receptor alpha (PPARA), and serum response factor (SRF)]. No targets were identified by IPA for the remaining 14 miRNAs (Table 4).

Gene Ontology prediction of targeted mRNAs in BROC intervention

Similarly, the PANTHER over-representation tool was utilized to identify biological processes and molecular functions that were significantly over-represented by the predicted mRNA targets in the BROC group. Table 7 and Table 8 display the top 15 enriched biological processes and the top 15 molecular functions of predicted mRNAs in broccoli-fed infants. Eight significantly over-represented mRNA biological processes had a fold enrichment greater than 100, with $p < 0.001$. In particular, 'Negative regulation of natural killer cell differentiation involved in immune response' and 'Regulation of natural killer cell differentiation', with identified mRNAs peptidoglycan recognition protein 2 (PGLYRP2) and peptidoglycan

recognition protein 1 (PGLYRP1), were found to be over-represented (Table 7). Additionally, three over-represented mRNA molecular functions had a fold enrichment greater than 100, with $p < 0.001$; two molecular functions significantly over-represented, were involved with interleukin-1 activity. These included 'Interleukin-1, type I, activating receptor activity' with identified mRNAs IL1R1, IL1RAPL2, and 'Interleukin-1 receptor activity' with the identified mRNAs being IL1RAP, IL1R1, IL1RL, and IL1RAPL2. Lastly, 'Interleukin-1 receptor binding' was over-represented with a fold enrichment of 94.35 and a $p<0.001$; identified mRNAs from our dataset for 'Interleukin-1 receptor binding' included TLR9, IL1RAP, IL36A, ILF10, IL36G, IL37, interleukin 36 receptor antagonist (IL36RN), and IL36B (Table 8).

Chapter 4

Discussion

A global miRNA profiling analysis was conducted to determine effects of short-term feeding of broccoli and carrot on infants' fecal miRNAs. In both CRT and BROC intervention, miR-146a-5p was upregulated and was predicted to target 80 mRNAs per the IPA target prediction algorithm (Table 3 and Table 4). Functional characteristics of these predicted target mRNAs include tumor suppression, immune response, and inflammatory regulation.

Regarding the immune response, the miR-146a-5p predicted to targeted signaling pathway is involved with TLR4; it is well established that the TLR4 signaling pathway plays an important role in activating the innate immune response [122]. Similarly, TLR1 was a predicted target by miR-146a-5p, which plays a critical role in pathogen recognition as well activating innate immunity [123]. Additionally, CRT also upregulated miR-130a-3p, which was predicted to target CSF1 (Table 3); CSF1 is critical for innate immunity and inflammatory processes through its capability to promote proinflammatory chemokines [123]. These three predicted target mRNAs (i.e., TLR1, TLR4, and CSF1) show the potential for dietary supplementation of carrots to increase the innate immunity in infants. This is particularly important for infant participants, as an increase in innate immunity may decrease the rate of early infection early on [124; 125; 126].

Other immune functions related miR-146a-5p's target mRNAs include C-reactive protein (CRP), complement factor H (CFH), component of inhibitor of NF-κB kinase complex (CHUK), CD40 molecule (CD40), and TNF receptor associated factor 6 (TRAF6). CRP supports host defense through recognition of pathogens and damaged cells of the host and to initiate their elimination while CFH protein regulates host immune response through destroying pathogens, initiation of inflammatory responses, and removal of debris from cells and tissues [123]. In

addition, CHUK (a target mRNA of miR-146a-5p) is known to participate in the canonical IKK complex by NF-κB activation and phosphorylates inhibitors of NF-κB on serine residues. NF-κB transitions to the nucleus and activates the transcription of hundreds of genes involved with immune response, growth control, or protection against apoptosis [123]. CD40 protein is responsible for mediating a variety of immune and inflammatory responses; including T-cell dependent immunoglobulin class switching, memory B cell development, and germinal center formation [123]. Last, TRAF6 may mediate CD40 signal, thus proving critical in immunoglobulin production regulation [123]. This evidence in literature collectively proposes that carrot supplementation may aid in an infant's capability to fight off disease after exposure, and could benefit the infant's immunity. As an upregulation of miR-146a-5p is predicted to impact these genes directly, it is yet to be seen the reach of beneficial impacts enacted by these targeted mRNAs.

CRT intervention may be related with tumor suppression as well. The miR-146a-5p (which was upregulated by the CRT) was predicted to target mRNAs, C-C motif chemokine ligand 8 (CCL8), BRCA1 DNA repair associated (BRCA1), and cyclin dependent kinase inhibitor 3 (CDKN3). The role of CCL8 still remains relatively unknown, however an increase of CCL8 expression has been linked to multiple cancers (e.g., squamous cell [127], and cervical [128]). In contrast, BRCA1 is well known to provide instructions for tumor suppression proteins, therefore aiding in the maintenance of the rate of cell proliferation [123]. Lastly, CDKN3 is related with cell cycle regulation and is known to be abnormally expressed (e.g., deleted, mutated, or overexpressed) in multiple cancers [123; 129; 130]. In addition, in the context of cancer, predicted target mRNAs of miR-147a-5p include MCL1 apoptosis regulator, BCL2 family member (MCL1). MCL1 inhibits apoptosis in isoform 1, and its overexpression has been linked to lung

cancer progression and decrease survival rates [123; 131; 132]. The miR-200c-3p, downregulated in the CRT intervention, was predicted to target BRCA1 associated protein 1 (BP1) and PTEN. Both of which act a tumor suppressors, regulating cell proliferation [123]. As these predicted target mRNAs are related with tumor development as well as progression (e.g., cell proliferation), carrot supplementation has the potential to aid in preventing growth of tumors as infants' bodies continue to grow and mature.

Lastly, when comparing CRT to CON, there were multiple targeted mRNAs involved in inflammatory processes through miRNA upregulation and downregulation. Upregulation of miR-146a-5p by CRT intervention was predicted to target multiple interleukins and cytokines (Table 3), as well as other supportive mRNAs [e.g., chemokine receptor 3(CCR), Fas associated via death domain (FADD), interferon regulatory factor 5 (IRF5), nitric oxide synthase 2 (NOS2), and signal transducer and activator of transcription 1 (STAT1)]. FADD, IRF5, and NOS2 are involved in the synthesis and development of various interleukins and T-cells [123]. Targeted mRNA CCR is associated with acute inflammatory response [133]. STAT1 is associated with the IL-17 pathway, and interferon-alpha/beta and interferon-gamma signaling pathways. These pathways are critical in the immune defense against bacteria and viruses [123]. Additionally, the upregulation of miR-130a-3p was predicted to target CSF1, which promotes the release of proinflammatory chemokines [123]. These results suggestion the role of key miRNAs influenced by CRT may play in regulating inflammation, which is further supported by the previously established anti-inflammatory effects of apiaceous vegetables, such as carrots [110; 134].

PANTHER analysis identified multiple biological processes and molecular functions, changed in the CRT intervention. Specific to immune function, the biological process 'Positive regulation of interleukin-12 biosynthetic process' had a significant fold change, and was

predicted to be influenced by mRNAs, TRAF6, LBT, TLR4. Interleukin 12 has an essential role in promoting T helper cell responses, therefore is found in cell-mediated immunity [135]. Likewise, many predicted molecular functions support these immunological processes potentially influenced by CRT intervention. For example, the predictions of 'Interleukin-1, type I, activating receptor activity' targeted by IL1R1 and IL1RL2, 'Interleukin-1 receptor activity' targeted by IL1RAP, IL1R1, IL1RL2, and IL1RAPL2, and 'Interleukin-1 receptor binding' targeted by TLR9, IL1RAP, IL36A, ILF10, IL36G, IL37, IL36RN, and IL36B. The interleukin 1 family (e.g., IL18, IL36, IL37, IL38) have been recognized for their pivotal roles in inflammation and immunity. Specifically, Interleukin 1 family members participates in therapeutic and preventative measures in autoimmunity and auto-inflammation [136]. These predicted biological processes and molecular functions display the potential of CRT to influence inflammatory processes.

Additionally, multiple biological processes involving macrophages were predicted to be influenced. Biological processes 'Positive regulation of cellular response to macrophage colony-stimulating factor stimulus,' Positive regulation of response to macrophage colony-stimulating factor,' 'Regulation of response to macrophage colony-stimulating factor,' and 'Regulation of cellular response to macrophage colony-stimulating factor stimulus' were predicted to be targeted by mRNAs, CSF1 and TLR4. Macrophages are central to the innate immune system, and plays a role in inflammation through release of cytokines [137]. The prediction of these four macrophage biological processes are supported by the prediction of cytokine molecular functions. Molecular function 'Cytokine receptor activity' was related with IL12RN2, IL1RAP, CCR3, CXCR4, IL1R1, IL1RL2, and IL1RAPL2. Collectively, these biological processes and

molecular functions suggest the potential of CRT intervention to impact the human infants' innate immunity.

In the BROC intervention, we found a total of 20 miRNAs differentially expressed in fecal samples. Unique to the BROC intervention, we observed a downregulation in miR-205-5p. When subjected to IPA analysis, predicted targeted mRNAs of miR-205-5p included ATP1A1, INPPL1, and PTEN; as previously discussed, PTEN is a tumor suppressor (which can target by miR-200c-3p in the CRT intervention). This is unsurprising, as miRNAs have multiple targets, and multiple miRNAs can target a single mRNA [138]. The three mRNAs predicted to be targeted by miR-205-5p proposes broccoli's potential capability to impact multiple systems within the body (i.e., Na+/K+ ATPase protein pump, insulin regulation, and tumor suppression), thereby the potential to have protective or therapeutic effects on multiple diseases (e.g., cancer, blood pressure, diabetes). ATP1A1 is a gene that produces one part of Na+/K+ ATPase protein pump; this pump is particularly important in adrenal glands and influences blood pressure in the body [123]. Additionally, INPPL1 encodes SH2-containing 5'-inositol phosphatase, which aids in the regulation of insulin [123]. Together, these target mRNAs show the potential of miRNAs supporting broccoli's impact on metabolic diseases (e.g., diabetes, hypertension) after phytonutrient absorption by the gut microbiome [139; 140]. As childhood metabolic diseases are on the rise world-wide, the beneficial effects of INPPL1 and ATP1A1 on insulin regulation and blood pressure may aid in the health of infants through dietary interventions. This would be an important next step for future studies, in order to fully understand the reach of BROC intervention of the long-term health of an infant.

Lastly, when BROC intervention had a predicted increase in immunoregulatory mRNAs, indicating a potential role in the immune system of the infant. Of note, the BROC also

upregulated miR-146a-5p, which targeted a total of 80 mRNA when subjected to IPA. As expected, the predicted target mRNAs were identical with those identified in the CRT intervention (e.g., TLR4, TLR1, CSF1, CRP, CFH, CHUK, CD40, and TRAF6) and many are discussed in detail above. Of note, IRAK1 is a putative serine/theonine kinase that is associated with interleukin-1 receptor, which is critical in innate immune response against pathogens [123]. Additionally, CCR, FADD, IRF5, NOS2, and STAT1, indicate potential impacts of broccoli supplementation on inflammatory responses. In addition, the BROC group showed decreased expression of miR-361-5p when compared to the CON, which was predicted to target activation induced cytidine deaminase (AICDA). AICDA is a protein involved in somatic hypermutation, gene conversion, and class-switch recombination of immunoglobulin genes [123]. Therefore, miR-361-5p may play a role in helping the immune system adapt to foreign elements through targeting AICDA. Although broccoli's influence on inflammation is well studied, this is the first time it has been predicted in human infants.

In the PANTHER analyses, BROC intervention recognized the enrichment of biological processes: 'Negative regulation of natural killer cell differentiation involved in immune response,' 'Negative regulation of natural killer cell differentiation,' and 'Regulation of natural killer cell differentiation involved in immune response.' Natural killer cells are vital in our body's defense system, and are utilized to attack infections and the growth of tumors [141]. As natural killer cell differentiation depends on T and B cells [142], multiple molecular functions identified support this process; For example, 'Lipopolysaccharide immune receptor activity' is related with mRNAs TLR4 and PTAFR. The presence of bacterial lipopolysaccharide activates natural killer cells [143], where the natural killer cells can then attack the foreign invaders. Additionally, lipopolysaccharide is a stimulator of monokines, including interleukin 1 [143].

Molecular functions, enriched in the BROC group, associated include 'Interleukin-1, type I, activating receptor activity' (associated with IL1R1 and IL1RL2), 'Interleukin-1 receptor activity' (associated with IL1RAP, IL1R1, IL1RL, and IL1RAPL2), and 'Interleukin-1 receptor binding' (associated with TLR9, IL1RAP, IL36A, IL36G, IL37, IL36RN, and IL36B). These predicted biological functions and molecular processes further suggest the potential influence of BROC intervention to immunity in human infants.

Although we were able to predict the impacts of CRT and BROC intervention on human infants' immunity, there are multiple areas within this research that need to be further explored. In the future, validation of miRNAs and mRNAs expression, using more specific experimental technique (e.g., quantitative PCR analysis) after BROC and CRT intervention would be informative. Additionally, studies with larger sample sizes could increase accuracy and provide better statistical power. We had a sample size of 11 infants, which was a challenge to recruit. Northwest Arkansas is a collective of multiple, relatively small towns and cities, which had trouble accepting the invitation for infant participants. We were limited in willing participants, age-range of participants, and timeframe of the study. In a more populated area, and with more time, there is a possibility for a large sample size of infants to conduct the study. Lastly, we were not able to perfectly match macro/micronutrients/fiber contents of the carrot and broccoli purée. However, both broccoli and carrot contained similar fiber content (BROC = 1.5 g; CRT = 1.6 g). Given the small differences between them, the fiber intake might have been impacted more by other factors such as spillage.

While there are a few limitations to our study, there are a few strengths. To our knowledge, our study is the first attempt that comprehensively profiled fecal miRNAs in response to CRT and BROC intervention in infant subjects. Additionally, we predicted mRNAs these miRNAs

influence through the IPA. In this, we were able to predict the extended potential effects of these miRNAs may have on the human infants. After, we exposed the vast amounts of predicted mRNAs to the PANTHER, predicting further into which biological processes and molecular functions may be impacted. As a result, this allows us to see the potential full affect CRT and BROC intervention may have on the health of human infants. Lastly, we were able to show that BROC and CRT intervention both have potential beneficial effects on infant immunity and, therefore, may help fight against common infant related infections.

Conclusion

In conclusion, we observed 22 miRNAs differentially expressed in CRT intervention, and 20 miRNAs differentially expressed in BROC intervention. When exposed to IPA and PANTHER, these differentially expressed miRNAs revealed a potential to impact multiple immune related functions. For example, miR-146a-5p in CRT intervention was predicted to target TLR4, which may influence biological processes involved with innate and acquired immunity (e.g., Positive regulation of interleukin-12 biosynthetic process). Additionally, miR-130a-3p in CRT intervention was predicted to target CSF1, which potentially impacts the innate immune system through CSF1 involvement in multiple biological processes involving macrophages. Comparatively, miR-146a-5p in BROC intervention was predicted to target PGLYRP2 and PGLYRP1. These mRNAs play a role in natural killer cells differentiation, therefore aiding in the immune systems capability to fight foreign invaders. Overall, our study provides potential evidence that CRT and BROC intervention impacts human infants' miRNAs, and may lead to overall benefits in their immune system.

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Appendix

Table 1. Searched phytochemicals subcategories, sources, and references.[1]

Phytochemicals			
Category:	*Subcategory:*	*Source:*	*Reference*
Flavonoids	Anthocyanidins	Acai Berry, Apple, Avocado, Banana, Blackberry, Blueberry, Cherries, Cranberry, Currants, Dates, Elderberry, Eggplant, Grapes, Grapefruit, Kiwifruit, Lingonberry, Pomegranate, Pear, Plum, Nuts, Raspberry, Strawberry, Tasmanian Peppers, Wine	(144)
	Flavone -3-ols	Apricots, Blackberry, Blueberry, Cranberry, Coffee, Chocolate, Grape Seeds, Kiwifruit, Nectarines, Nuts, Peaches, Plums, Rhubarb, Tea, Wine	(144)
	Flavones	Artichokes, Celery Seed Spice, Celery, Chicory, Honey, Juniper berry, Kumquats, Oregano, Olive Leaves, Pumpkin, Parsley, Pimento Peppers, Sweet Peppers, Sage, Thyme,	(144)
	Flavonols	Arugula, Asparagus, Bay Leaves, Capers, Cilantro, Chives, Cherries, Cranberries, Dill Weed, Elderberries, Juniper Berry, Okra, Parsley, Plum, Prickly, Pears, Saffron, Tarragon	(144)
	Isoflavones	Miso, Natto, Soy, Tempeh, Tofu	(145)
Carotenoids	Carotenes (pro-vitamin A)	Apricot, Brussel Sprouts, Cantaloupe, Cilantro, Kale, Romain Lettuce, Mango, Orange Pepper, Spinach, Butternut Squash, Watermelon	(146)
	Xanthopylls (non-pro vitamin A)	Artichoke, Asparagus, Broccoli, Egg, Kale, Romain Lettuce, Pistachio Nuts, Parsley, Red Pepper, Scallions, Spinach, Zucchini	(146)
Glucosinolates	Isothiocyanates	Broccoli, Brussel Sprouts, Cauliflower, Cabbage, Kale, Turnips	(147)
Phytosterols	Epicholesterol	Nuts, Seeds	(148)
	6-Ketochilestanol	Walnuts, Almonds, Peanuts, Hazelnuts, Macadamia Nuts	(148)
	Cholesteryl	Vegetable Oils, Spelt and Wheat Cereals	(148)
Allicins	Organosulfur Compounds	Garlic, Onions, Leeks	(149) (150)

[1]The databases PubMed and Google Scholar were utilized for the literature search.

Figure 1. Food Recall Example

24 Hours Food Record (Day 1)

Preparer:

Date:

Time	Food Items (Breast milk, formula or others?)	Approximate Amount (Duration if Breastfeeding)

Day 1 is the first intervention day.

Figure 2. Study Design Example

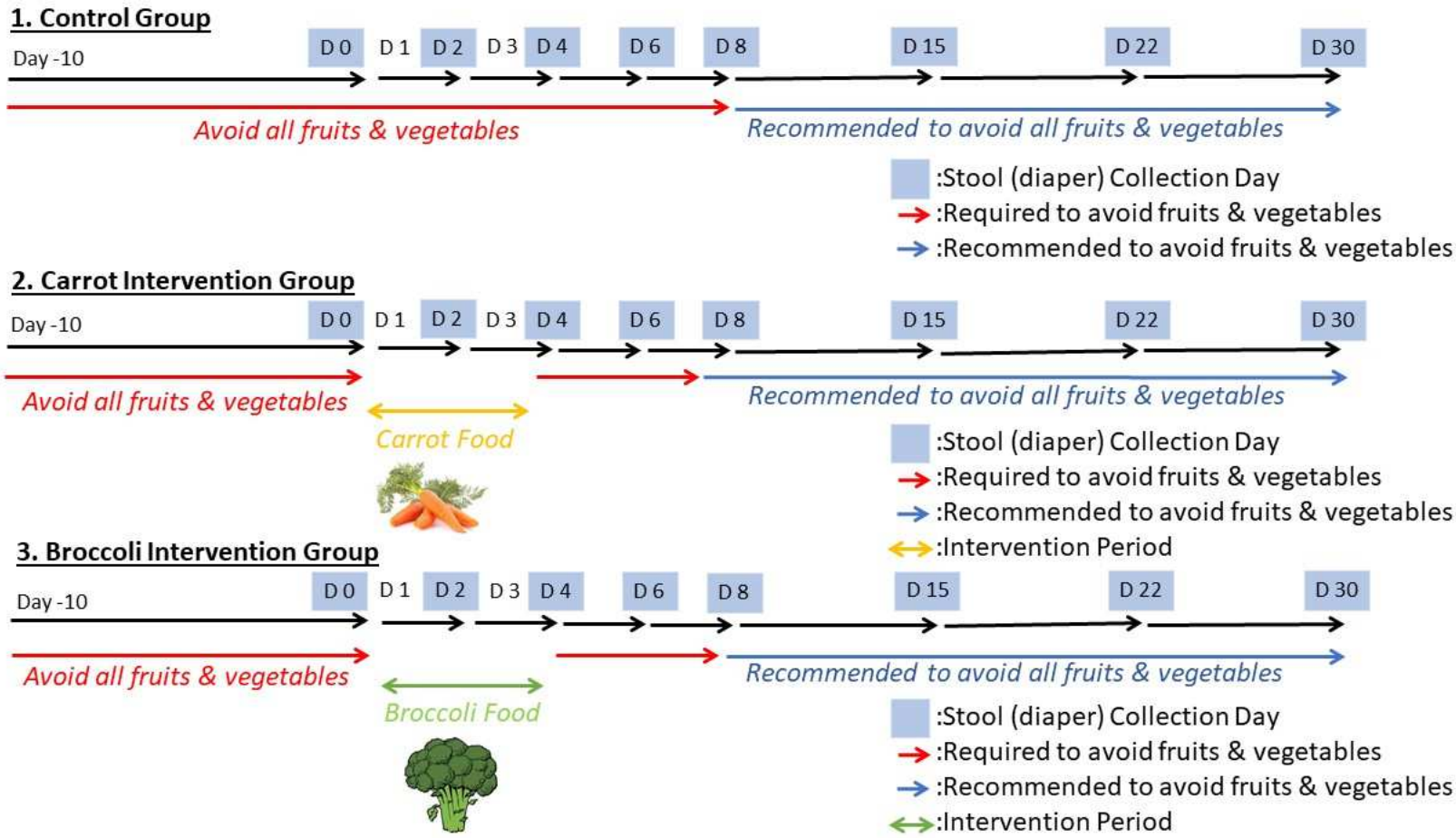

Table 2. Infant participant demographic

	Category	Number of Participants
Ethnicity	Asian	1
	White	3
	Native Indian or Alaskan Native & White	1
	Asian & White	1
	Non -Hispanic or Latino & White	5
Milk Source	Breastfed	8
	Formula Fed	3
	Both	1

Table 3. Effects of carrot intervention on miRNA

Carrot DEmiR (1.5 Folds; p<0.05)[1]				
miRNA symbol	miRBase accession	Avg RQ ± SEM	P-value vs control	Target mRNA[2]
hsa-miR-1253	MIMAT0005904	1.65 ± 0.14	0.017	Not Predicted
hsa-miR-130a-3p	MIMAT0000425	4.85 ± 0.59	0.004	ATG2B, CSF1, DICER1, HOXA5, MAFB, MEOX2, SMAD4, TAC1, ZFPM2
hsa-miR-146a-5p	MIMAT0000449	2.38 ± 0.28	0.016	ATOH8, BLMH, BRCA1, C8A, CAMP, CCL8, CCNA2, CCR3, CD1D, CD40, CDKN3, CFH, CHUK, COL13A1, CRP, CXCL8, CXCR4, DMBT1, FADD, IFNA1/IFNA13, IFNB1, IL10, IL12RB2, IL1F10, IL1R1, IL1RAP, IL1RAPL2¸ IL1RL2, IL36A, IL36B, IL36G, IL36RN, IL37, IRAK1, IRAK2, IRF5, KIF22, KIR2DL1/KIR2DL3, KIR2DL2, LALBA, LBP, LTB, LTF, MCM10, MCPH1, METTL7A, MMP16, MR1, NFIX, NLGN1, NOS2, NOVA1, PA2G4, PBLD, PDGFRA, PDIK1L, PEX11G,PGLYR1, PGLYP2, PLEKHA4, POLE2, PRR15, PTAFR, PTGES2, RAD54L, S100A12, SDCBP2, SFTPD, STAT1, SYT1, TIMELESS, TLR1, TLR10, TLR4, TLR9, TMSB15A, TRAF6, TRIM14, UHRF1, VWCE
hsa-miR-147a	MIMAT0000251	2.19 ± 0.36	0.043	VEGFA
hsa-miR-193a-3p	MIMAT0000459	2.11 ± 0.21	0.029	CCND1, E2F6, ERBB4, ESR1, MCL1, PLAU, PTK2, RPS6KB2
hsa-miR-200b-3p	MIMAT0000617	0.38 ± 0.05	0.004	ACE2, BAP1, ELMO2, ERBIN, ERRFI1, FHOD1, FOXF2, GEMIN2, GSE1, KLHL20, MARCKS, PLCG1, PPM1F, PTEN, PTPN12, PTPN13, PTPRD, RERE, WASF3, WDR37, ZEB1, ZEB2, ZFPM2
hsa-miR-2116-5p	MIMAT0011160	0.35 ± 0.11	0.047	Not Predicted
hsa-miR-300	MIMAT0004903	0.61 ± 0.10	0.034	Not Predicted
hsa-miR-3127-5p	MIMAT0014990	3.13 ± 0.37	0.010	Not Predicted
hsa-miR-3144-3p	MIMAT0015015	1.98 ± 0.22	0.024	Not Predicted
hsa-miR-320a-3p	MIMAT0000510	0.56 ± 0.11	0.046	HSPB6, TAGLN, VIM
hsa-miR-365b-5p	MIMAT0022833	2.84 ± 0.48	0.031	Not Predicted
hsa-miR-374c-5p	MIMAT0018443	2.15 ± 0.36	0.050	Not Predicted
hsa-miR-376b-3p	MIMAT0002172	2.94 ± 0.58	0.045	Not Predicted
hsa-miR-378b	MIMAT0014999	0.13 ± 0.02	0.035	CASP9, IGF1R, ODC1, PDPK1, SUFU, TUSC2
hsa-miR-508-5p	MIMAT0004778	0.44 ± 0.16	0.036	Not Predicted
hsa-miR-521	MIMAT0002854	0.18 ± 0.05	0.048	ERCC8
hsa-miR-561-5p	MIMAT0022706	2.06 ± 0.31	0.038	Not Predicted
hsa-miR-595	MIMAT0003263	2.81 ± 0.49	0.033	Not Predicted
hsa-miR-603	MIMAT0003271	2.90 ± 0.26	0.002	Not Predicted
hsa-miR-627-5p	MIMAT0003296	2.19 ± 0.27	0.040	Not Predicted
hsa-miR-885-3p	MIMAT0004948	0.27 ± 0.13	0.005	Not Predicted

[1]Nanosting [2]Ingunitive Pathway Analysis (IPA)

Table 4. Effects of broccoli intervention on miRNA

Broccoli DEmiR (1.5 Folds; p<0.05)[1]				
miRNA symbol	miRBase accession	Avg RQ ± SEM	P-value vs control	Target mRNA[2]
hsa-miR-146a-5p	MIMAT0000449	3.07 ± 0.56	0.037	ATOH8, BLMH, BRCA1, C8A, CAMP, CCL8, CCNA2, CCR3, CD1D, CD40, CDKN3, CFH, CHUK, COL13A1, CRP, CXCL8, CXCR4, DMBT1, FADD, IFNA1/IFNA13, IFNB1, IL10, IL12RB2, IL1F10, IL1R1, IL1RAP. IL1RAPL2, IL1RL2, IL36A, IL36B, IL36G, IL36RN, IL37, IRAK1, IRAK2, IRF5, KIF22, KIR2DL1/KIR2DL3, KIR2DL2, LALBA, LBP, LTB, LTF, MCM10, MCPH1, METTL7A, MMP16, MR1, NFIX, NLGN1, NOS2, NOVA1, PA2G4, PBLD, PDGFRA, PDIK1L, PEX11G, PGLYRP1, PGLYRP2, PLEKHA4, POLE2, PRR15, PTAFR, PTGES2, RAD54L, S100A12, SDCBP2, SFTPD, STAT1, SYT1, TIMELESS, TLR1, TLR10, TLR4, TLR9, TMSB15A, TRAF6, TRIM14, UHRF1, VWCE
hsa-miR-190b	MIMAT0004929	2.66 ± 0.46	0.035	Not Predicted
hsa-miR-205-5p	MIMAT0000266	0.45 ± 0.10	0.038	ATP1A1, DOK4, ERBB3, INPPL1, MED1, PRKCE, PTEN, TRPS1, VEGFA, ZEB1, ZEB2
hsa-miR-2116-5p	MIMAT0011160	0.32 ± 0.06	0.015	Not Predicted
hsa-miR-22-3p	MIMAT0000077	2.80 ± 0.44	0.034	BMP7, ESR1, MAX, PPARA, SRF
hsa-miR-329-3p	MIMAT0001629	0.38 ± 0.10	0.017	Not Predicted
hsa-miR-361-5p	MIMAT0000703	0.21 ± 0.06	0.004	AICDA
hsa-miR-3613-3p	MIMAT0017991	0.35 ± 0.05	0.008	Not Predicted
hsa-miR-3934-5p	MIMAT0018349	2.82 ± 0.44	0.025	Not Predicted
hsa-miR-409-5p	MIMAT0001638	2.31 ± 0.30	0.036	Not Predicted
hsa-miR-4425	MIMAT0018940	0.34 ± 0.06	0.035	Not Predicted
hsa-miR-4443	MIMAT0018961	0.63 ± 0.08	0.013	Not Predicted
hsa-miR-499a-3p	MIMAT0004772	0.45 ± 0.12	0.034	Not Predicted
hsa-miR-503-3p	MIMAT0022925	0.34 ± 0.07	0.003	Not Predicted
hsa-miR-520f-3p	MIMAT0002830	2.79 ± 0.41	0.022	Not Predicted
hsa-miR-548i	MIMAT0005935	1.97 ± 0.25	0.048	ERBB2, MTA1
hsa-miR-573	MIMAT0003238	3.85 ± 0.84	0.047	Not Predicted
hsa-miR-579-5p	MIMAT0026616	0.54 ± 0.11	0.032	Not Predicted
hsa-miR-596	MIMAT0003264	0.22 ± 0.09	0.027	Not Predicted
hsa-miR-612	MIMAT0003280	1.65 ± 0.17	0.031	AKT2, TP53

[1]Nanosting Analysis Results [2]Ingunitive Pathway Analysis (IPA) Target mRNA.

Table 5. Signaling prediction of biological process for short-list miRNA in carrot-fed infants

Panther GO Biological Process Terms	N genes in reference genome dataset	N genes in dataset	Target mRNA identified in dataset	Fold Enrichment	P-value
Negative regulation of natural killer cell differentiation involved in immune response	3	2	PGLYRP2, PGLYP1	>100	< 0.001
Negative regulation of natural killer cell differentiation	3	2	PGLYRP2, PGLYP1	>100	< 0.001
Positive regulation of cell proliferation by VEGF-activated platelet derived growth factor receptor signaling pathway	3	2	VEGFA, PDGFRA	>100	< 0.001
VEGF-activated platelet-derived growth factor receptor signaling pathway	3	2	CSF1, TLR4	>100	< 0.001
Growth of symbiont in host	3	2	PGLYRP2, PGLYP1	>100	< 0.001
Growth involved in symbiotic interaction	3	2	PGLYRP2, PGLYP1	>100	< 0.001
Positive regulation of cellular response to macrophage colony-stimulating factor stimulus	3	2	CSF1, TLR4	>100	< 0.001
Positive regulation of response to macrophage colony-stimulating factor	3	2	CSF1, TLR4	>100	< 0.001
Regulation of natural killer cell differentiation involved in immune response	4	2	PGLYRP2, PGLYP1	76.66	< 0.001
Receptor biosynthetic process	5	2	ACE2, IL10	61.33	< 0.001
Regulation of cellular response to macrophage colony-stimulating factor stimulus	5	2	CSF1, TLR4	61.33	< 0.001
Regulation of response to macrophage colony-stimulating factor	5	2	CSF1, TLR4	61.33	< 0.001
Detection of bacterial lipoprotein	5	2	DMBT1, TLR1	61.33	< 0.001
Positive regulation of interleukin-12 biosynthetic process	8	3	TRAF6, LBT, TLR4	57.49	< 0.001
Negative regulation of tumor necrosis factor biosynthetic process	8	3	IL37, IL10, ERRFI1	57.49	< 0.001

Table 6. Signaling prediction of molecular function for short-list miRNA in carrot-fed infants

Panther GO Molecular Function Terms	N genes in reference genome dataset	N genes in dataset	Target mRNA identified in dataset	Fold Enrichment	P-value
Interleukin-1, type I, activating receptor activity	2	2	IL1R1, IL1RL2	>100	< 0.001
Interleukin-1 receptor activity	7	4	IL1RAP, IL1R1, IL1RL2, IL1RAPL2	87.61	< 0.001
Interleukin-1 receptor binding	17	8	TLR9, IL1RAP, IL36A, ILF10, IL36G, IL37, IL36RN, IL36B	72.15	< 0.001
NAD+ nuleotidase, cyclic ADP-ribose generating	16	7	IL1RAP, IL1R1, TLR4, TLR10, IL1RL2, IL1RAPL2, TLR1	67.08	< 0.001
NAD+ nucleosidase activity	16	7	IL1RAP, IL1R1, TLR4, TLR10, IL1RL2, IL1RAPL2, TLR1	67.08	< 0.001
NAD(P)+ nucleosidase activity	16	7	IL1RAP, IL1R1, TLR4, TLR10, IL1RL2, IL1RAPL2, TLR1	67.08	<0.001
Lipopeptide binding	10	3	TLR1, LBP, CD1D	45.99	< 0.001
Platelet-derived growth factor receptor binding	15	4	PTEN, VEGFA, PDGFRA, IL1R1	40.88	< 0.001
Pattern recognition receptor activity	24	6	TLR9, DMBT1, PGLYRP2, TLR4, PTAFR, PGLYRP1	38.33	< 0.001
Hydrolase activity, hydrolyzing N-glycosyl compounds	38	7	IL1RAP, IL1R1, TLR4, TLR10, ILRL2, ILRAP2, TLR1	28.24	< 0.001
Lipopolysaccharide binding	33	6	CAMP, DMBT1, TLR4, PTAFR, LTF, LBP	27.88	< 0.001
Tumor necrosis factor receptor binding	32	4	TRAF6, STAT1, LTB, FADD	19.16	< 0.001
Growth factor receptor binding	136	14	TRAF6, IL1RAP, IL36A, IL1F10, IL36G, PTEN, IL37, VEGFA, PDGFRA, ILR1, ERBB4, IL10, IL36RN, IL36B	15.78	< 0.001
Tumor necrosis factor receptor superfamily binding	49	4	TRAF6, STAT1, LTB, FADD	12.52	< 0.001
Cytokine receptor activity	99	7	IL12RN2, IL1RAP, CCR3, CXCR4, IL1R1, IL1RL2, IL1RAPL2	10.84	< 0.001

Table 7. Signaling prediction of biological processes for short-list miRNA in broccoli-fed infants

Panther GO Biological Process Terms	N genes in reference genome dataset	N genes in dataset	Target mRNA identified in dataset	Fold Enrichment	P-value
Metanephric mesenchymal cell proliferation involved in metanephros development	3	2	BMP7, STAT1	>100	<0.001
Kidney mesenchymal cell proliferation	3	2	BMP7, STAT1	>100	<0.001
Negative regulation of natural killer cell differentiation involved in immune response	3	2	PGLYRP2, PGLYRP1	>100	<0.001
Negative regulation of natural killer cell differentiation	3	2	PGLYRP2, PGLYRP1	>100	<0.001
Positive regulation of cell proliferation by VEGF-activated platelet derived growth factor receptor signaling pathway	3	2	VEGFA, PDGFRA	>100	<0.001
VEGF-activated platelet-derived growth factor receptor signaling pathway	3	2	VEGFA, PDGFRA	>100	<0.001
Growth of symbiont in host	3	2	PGLYRP2, PGLYRP1	>100	<0.001
Growth involved in symbiotic interaction	3	2	PGLYRP2, PGLYRP1	>100	<0.001
Regulation of natural killer cell differentiation involved in immune response	4	2	PGLYRP2, PGLYRP1	99.29	<0.001
Epithelial cell proliferation involved in mammary gland duct elongation	4	2	MED1, ESR1	99.29	<0.001
Detection of bacterial lipoprotein	5	2	DMBT1, TLR1	79.43	<0.001
Branch elongation involved in mammary gland duct branching	4	2	MED1, ESR1	79.43	<0.001
Positive regulation of interleukin-12 biosynthetic process	7	3	TRAF6, LTB, TLR4	74.47	<0.001
Detection of other organism	14	5	PGLYRP2, TLR4, PGLYRP1, TLR1, CD1D	70.09	<0.001
Regulation of interferon-alpha biosynthetic process	6	2	TLR9, IL10	70.09	<0.001

Table 8. Signaling prediction of molecular function for short-list miRNA in broccoli-fed infants

Panther GO Molecular Function Terms	N genes in reference genome dataset	N genes in dataset	Target mRNA identified in dataset	Fold Enrichment	P-value
Interleukin-1, type I, activating receptor activity	2	2	IL1R1, IL1RAPL2	>100	< 0.001
Interleukin-1 receptor activity	7	4	IL1RAP, IL1R1, IL1RL, IL1RAPL2	>100	< 0.001
N-acetylmuramoyl-L-alanine amidase activity	4	2	PGLYP1, PGLYP2	>100	< 0.001
Interleukin-1 receptor binding	17	8	TLR9, IL1RAP, IL36A, IL36G, IL37, IL36RN, IL36B	94.35	< 0.001
NAD+ nucleosidase, cyclic ADP-ribose generating	16	7	IL1RAP, IL1R1, TLR4, TLR10, IL1RL2, IL1RAPL2, TLR1	86.88	<0.001
NAD+ nucleosidase activity	16	7	IL1RAP, IL1R1, TLR4, TLR10, IL1RL2, IL1RAPL2, TLR1	86.88	<0.001
NAD(P)+ nucleosidase activity	16	7	IL1RAP, IL1R1, TLR4, TLR10, IL1RL2, IL1RAPL2, TLR1	86.88	<0.001
Lipopolysaccharide immune receptor activity	5	2	TLR4, PTAFR	79.43	0.047
ErB-3 class receptor binding	5	2	ERBB3, ERBB2	79.43	0.046
Lipoteichoic acid binding	5	2	DMBT1, LBP	79.43	0.045
Peptidoglycan immune receptor regulator	5	2	PGLYP1, PGLYP2	79.43	0.045
Lipopeptide binding	10	3	TLR1, LBP, CD1D	59.57	0.004
Platelet-derived growth factor receptor binding	15	4	PTEN, VEGFA, PDGFRA, IL1R1	52.57	0.004
Pattern recognition receptor activity	22	6	TLR9, DMBT1, PGLYRP2, TLR4, PTAFR, PGLYRP1	49.65	< 0.001
Hydrolase activity, hydrolyzing N-glycosyl compounds	38	8	IL1RAP, MED1, IL1R1, TLR4, TLR10, IL1RL2, IL1RAPL2, TLR1	41.81	< 0.001

Supplementary Figure 1. IRB Approval Form

UNIVERSITY OF ARKANSAS

To:	Jae Kyeom Kim BELL 4188
From:	Chair, Douglas James Adams IRB Committee
Date:	05/30/2018
Action:	**Approval**
Action Date:	05/30/2018
Protocol #:	1710079880
Study Title:	Impact of glucosinolate-rich broccoli on gut microbiota, microRNA profile, and immune health in infants.
Expiration Date:	02/25/2019
Last Approval Date:	
Risk Level:	

The above-referenced protocol has been approved following Full Board Review by the IRB Committee that oversees research with human subjects.

If the research involves collaboration with another institution then the research cannot commence until the Committee receives written notification of approval from the collaborating institution's IRB.

It is the Principal Investigator's responsibility to obtain review and continued approval before the expiration date.

Protocol are approved for a maximum period of one year. You may not continue any research activity beyond the expiration date without Committee approval. Please submit continuation requests early enough to allow sufficient time for review. Failure to receive approval for continuation before the expiration date will result in the automatic suspension of the approval of this protocol. Information collected following suspension is unapproved research and cannot be reported or published as research data. If you do not wish continued approval, please notify the Committee of the study closure.

Adverse Events: Any serious or unexpected adverse event must be reported to the IRB Committee within 48 hours. All other adverse events should be reported within 10 working days.

Amendments: If you wish to change any aspect of this study, such as the procedures, the consent forms, study personnel, or number of participants please submit an amendment to the IRB. All changes must be approved by the IRB Committee before they can be initiated.

You must maintain a research file for at least 3 years after completion of the study. This file should include all correspondence with the IRB committee, original signed consent forms, and study data.

cc: Sabrina Trudo, Investigator
Mechelle Baily, Investigator
Jiangchao Zhao, Investigator
Allison L Scott, Investigator
Marilou D Shreve, Investigator
JungAe Lee-Bartlett, Investigator
Jeonghoon Pan, Key Personnel

Supplementary Figure 2. IRB Protocol Approval