EVALUATING MIR168A LEVELS IN PROCESSED POTATOES AND MAMMALIAN SERA

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ABSTRACT

MicroRNAs (miRNAs or miRs) are short, non-coding segments of RNA that regulate gene expression and are endogenously produced by plants and mammals. Recent evidence has indicated that exogenous plant microRNAs acquired through diet may have the ability to regulate mammalian gene expression in a phenomena termed cross-kingdom regulation. The results of these studies remain controversial, and it remains contested as to whether or not exogenous microRNAs can truly be absorbed into the mammalian serum via diet. Additionally, while these studies have evaluated the effect of exogenous, dietary plant microRNAs on mammalian gene expression, they have yet to examine the effects of processing on exogenous plant microRNA absorption. Consequently, this study aimed to evaluate the levels of a specific microRNA, miR-168a, in processed, namely baked and chipped, White Atlantic and Purple Majesty potatoes. Furthermore, this study also sought to evaluate the levels of miR-168a in the serum of pigs that had been fed these processed White Atlantic and Purple Majesty potatoes. It was thought that chipped samples of both potato varieties would show the greatest decrease in miR-168a levels in comparison to raw or baked samples. In addition, it was thought that pigs fed these potatoes would show increased serum levels of miR-168a in comparison to pigs fed a control diet. More specifically, it was thought that pigs fed chipped varieties of both potatoes would show the smallest increase in serum levels of miR-168a in comparison to pigs fed raw or baked varieties of both potatoes. Ultimately, this study showed that processing decreased levels of miR-168a in White Atlantic and Purple Majesty potatoes. Additionally, miR-168a was absorbed from White Atlantic and Purple Majesty potatoes into the serum of pigs fed those potatoes. It appeared that absorption was variety-dependent, and miR-168a was better absorbed into the serum of pigs fed
White Atlantic potatoes and opposed to Purple Majesty potatoes. While this study could not conclusively determine whether or not potato processing affected how well miR-168a was absorbed into pigs’ sera, it appears that chipping allowed for better absorption of miR-168a into sera than baking across both varieties of potatoes.
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Chapter 1
Introduction

1.1: Problem Statement

In the past few decades, microRNAs (also known as miRs or miRNAs) have become a centerpiece of discussion in the scientific community. These short, non-coding segments of RNA have the ability to mediate gene function and turn genes on and off. Produced endogenously in humans, microRNAs have been shown to manipulate the human genome in ways that change gene regulation, affect cell communication, and impact overall human health.\(^1\) However, the production of microRNAs is not unique to humans; plants and viruses also utilize microRNAs to regulate their own gene function. In fact, there are a handful of plant microRNAs that have shown potential to target meaningful sequences in the human genome. Accordingly, in recent years, the food science industry has begun to evaluate microRNAs' abilities to act as a bioactive compound that can cause relevant health effects in humans via the consumption of plant- and animal-based foods. Some core areas of interest in studying microRNAs in plant-based foods include understanding if microRNAs from plants can actually be assimilated by mammals via diet, if hypothetically assimilated plant microRNAs can regulate human genes, and if assimilation and plant microRNA levels are affected by food processing and types of food.

One microRNA that has been considered as a plant-based bioactive compound is miR168a. Mir-168a is found in a variety of plants and targets the sequence for human gene LDLRAP1, which regulates low-density lipoprotein levels (LDL) in the bloodstream.\(^2\) In a study published in 2012, researchers found that exogenous plant miR168a was assimilated into the
human bloodstream and affected LDLRAP1 gene function in those who consumed a diet high in rice. However, no additional work has been done to confirm the ability of miR168a to assimilate into the bloodstream via diet. Secondly, the role of miR168a as a bioactive compound in other plant-based foods where it is present has not been examined. Lastly, and perhaps most importantly, no work has been done to address the effects of food processing on miR168a levels and how the microRNA may assimilate into the mammalian bloodstream. Food processing can greatly impact the nutritional content of food, and it is valuable to study how processing could affect miR168a’s ability to act as a bioactive compound.

To address these gaps in knowledge, a thesis study was formulated to evaluate miR168a’s role as a bioactive compound in a new plant model - potatoes. Potatoes are the leading vegetable crop in the United States and represent 15% of all vegetable-based agriculture revenue. Additionally, nearly fifty percent of potato sales are for processed varieties like chips or baked products. Most importantly, miR168a is present in all species of potatoes. Consequently, potatoes present a highly relevant model to not only evaluate the ability of miR168a to assimilate into the bloodstream via consumption, but also to study the effects of processing on miR168a levels and the microRNA’s ability to assimilate into the mammalian bloodstream. This thesis study aimed to understand how levels of miR168a varied between two commonly consumed varieties of potatoes: White Atlantic and Purple Majesty. Additionally, this study examined how processing, namely baking and chipping, affected miR168a levels within each variety of potato and in the sera of pigs that had been fed raw, baked, and chipped potatoes of both varieties.
1.2: Hypothesis

It is expected that chipping will decrease miR168a levels more than baking in both varieties of potatoes. It is also expected that exogenous miR-168a from raw, baked, and chipped varieties of both potatoes will be absorbed into the sera of pigs fed those potatoes. Furthermore, it is expected that miR-168a levels will be lower in the sera of pigs fed chips of both potato varieties than pigs fed baked or raw potatoes of both varieties.

1.3: Objectives

- To extract microRNA from raw (control), baked, and chipped White Atlantic and Purple Majesty potatoes
- To extract microRNA from the sera of pigs fed raw (control), baked, and chipped White Atlantic and Purple Majesty potatoes
- To analyze levels of miR168a in raw (control), baked, and chipped White Atlantic and Purple Majesty potatoes via RT-PCR
- To analyze levels of miR168a from the sera of pigs fed raw (control), baked, and chipped White Atlantic and Purple Majesty potatoes via RT-PCR
Chapter 2
Review of Literature

2.1: The History, Function, and Mechanism of MicroRNAs

The study of microRNA is a fairly recent development in the scientific world. Thirty years ago, the scientific community knew nothing of microRNAs’ function or existence. It was only in 1993, due to the efforts of American biologists Gary Ruvkun and Victor Ambros, that the very first microRNA, lin-4, was discovered. At the time, it was known that lin-4 was a gene found in Caenorhabditis elegans that controlled the temporal development pattern of all larval stages. While trying to sequence lin-4, Ambros found that the transcript was very small (20-60 nt in length) and did not contain the normal stop or start codons found in gene transcripts. Upon further exploration, Ambros and Ruvkun realized that lin-4 was complementary to the lin-14 gene and controlled temporal development by binding and regulating lin-14. Through this discovery, Ruvkun and Ambros independently came to the conclusion that lin-4 was a microRNA and not a conventional gene transcript. These short (20-22 nucleotides in length), non-coding segments of RNA, have since been shown as critical posttranscriptional regulators of gene expression. MicroRNAs are found in a variety of organisms, including animals, plants, viruses, and protozoa. In mammals, it is thought that microRNAs regulate nearly 60% of protein-coding genes. Consequently, microRNAs have been shown to affect a wide variety of mammalian biological processes, including development, differentiation, proliferation, and stress responses.
In order to regulate gene expression, microRNAs repress protein synthesis. The mechanism by which microRNAs repress protein synthesis varies between organisms, though mechanisms between plants and animals are similar. Focusing on plants and animals, there are generally two ways that miRNAs have been shown to inhibit protein synthesis: mRNA degradation and translational repression. To inhibit protein synthesis via mRNA degradation, miRNAs form a ribonucleoprotein complex known as the miRNA-induced silencing complex (miRISC). Key components of the miRISC complex include a small RNA (microRNA) and one to multiple Argonaute (Ago) proteins. In animals, the microRNA of the miRISC partially base pairs with an mRNA targeted for degradation. The Ago proteins then recruit deadenylase complexes which remove the 3’ poly-A tail from the target mRNA. Following the removal of the poly-A tail, a decapping enzyme removes the 5’ methyl guanine cap from the target mRNA. Removal of the poly-A tail and the methyl guanine cap renders the target mRNA susceptible to a cytoplasmic exonuclease that finally degrades the target mRNA. It is valuable to note that Ago proteins recruit enzymes associated with a normal cellular pathway, the 5’-to-3’ decay pathway. This pathway is normally used to degrade excess mRNA. In plants, polyadenylation of the target mRNA does not occur. Instead, the microRNA of the miRISC complex completely base pairs with the target mRNA and the associated Ago protein cleaves the target mRNA. The 3’ and 5’ fragments of the cleaved target mRNA can then be degraded by different enzymes. The mechanism by which miRNAs regulate gene expression via mRNA degradation is fairly well understood, but the mechanism by which miRNAs regulate gene expression via translational repression is not. There are several proposed mechanisms on how miRNAs may inhibit translation, but overall this function of miRNAs remains poorly understood. In addition, it
seems that certain miRNAs may be able to activate translation, presenting another function of miRNAs that needs to be studied more intensely.\textsuperscript{5}

2.2: The Link Between Endogenous MicroRNAs and Human Disease: Cancer

Since microRNAs are multifunctional molecules that can affect many cellular pathways and cause diverse biological effects, it is logical that they have been implicated in many human health conditions. One disease that endogenous human microRNAs have been implicated in is cancer.

MicroRNAs have been shown to play a diverse role in cancer, and evidence has demonstrated that they have the ability to both promote and suppress cancer progression.\textsuperscript{5, 9} The role of microRNAs within cancer is diverse, complex, and sometimes contradictory. This can be demonstrated by looking at three microRNAs involved in cancer: the let-7 miRs, miR-21, and miR-9. For example, let-7 miRs have been shown to suppress cancer metastasis in colon cancer, melanoma, breast cancer, and gastric cancer models.\textsuperscript{9} On the other hand, miR-21 is associated with cancer promotion and metastasis in many cancers including lung cancer, gastric cancer, breast cancer, ovarian carcinoma, and bladder cancer.\textsuperscript{9} The difference in function between let-7 miRs and miR-21 shows that different microRNAs can serve opposing roles within many cancer models. However, the role of microRNAs can not only be opposing, but also contradictory. For example, miR-9 promotes gastric cancer but suppresses cancer proliferation in ovarian carcinoma.\textsuperscript{9} This illustrates that the function of a single microRNA can be altered based on the cancer it is involved in. Thus, as the list of microRNAs involved in various types of cancer continues to grow, it is important to be aware of their diversity in function.
Studies have shown that microRNAs can target many different levels of gene expression to both directly and indirectly cause cancer promoting and suppressing events. Therefore, different microRNAs have shown involvement in nearly all stages of cancer progression.\(^9\) However, there is an overwhelming body of evidence indicating that many cancer-involved microRNAs seem to target genes related to the epithelial-mesenchymal transition (EMT) in cancer metastasis.\(^9\) EMT is a cell program that results in epithelial cells taking on properties of mesenchymal cells.\(^9\) Mesenchymal cells are multipotent cells that have the ability to differentiate into a variety of cell lines.\(^9\) In addition, mesenchymal cells experience a loss of adhesion, increased polarity, and separation.\(^9\) Normally, EMT is involved in processes that require the rapid mobilization of cells, like wound healing and embryonic development.\(^9\) Consequently, EMT is often activated by cancer cells during metastasis to allow the cancer cells to move to new locations, shifting a tumor from benign to metastatic.\(^9\) Many microRNAs seem to target and affect genes related to the EMT cellular program. As seen before, different microRNAs can affect cancer progression differently. Likewise, different microRNAs can affect the EMT in cancer metastasis differently and in contradictory ways. For example, studies have shown that the miR-200 family of microRNAs, consisting of miR-200a, miR-200b, miR-200c, miR-141, and miR-429, is heavily involved in suppressing the EMT pathway in melanoma and squamous cell carcinoma.\(^9\) However, recent evidence has shown that one member of this family, miR-200c, has the ability to suppress tumor progression in renal cell carcinoma but promote cancer proliferation in breast cancer.\(^9,10\) Likewise, different microRNAs have been shown to both inhibit and promote EMT in a diverse array of cancers.\(^10,11,12,13,14,15,16\)

Ultimately, many microRNAs have been implicated in the progression and suppression of cancer in different models. Even individual microRNAs are susceptible to modulation by a
cancer microenvironment and can serve diverse functions. Specifically, EMT is a common cellular program targeted by microRNAs to both promote and suppress cancer.

2.3: The Link Between Endogenous MicroRNAs and Human Disease: Cardiovascular Disease

MicroRNAs have also shown activity in mitigating and aggravating cardiovascular disease, including, but not limited to, cardiac hypertrophy, cardiac fibrosis, and atherosclerosis.

Cardiac hypertrophy refers to the enlargement of the heart that occurs in response to stress. At its core, cardiac hypertrophy is an adaptive response to stress. However, if it is sustained for prolonged periods, cardiac hypertrophy can lead to heart failure, heart attacks, and death. Different microRNAs have been shown to exert functions that both suppress and promote cardiac hypertrophy. One of the most abundant cardiac microRNAs, miR-1, has been shown to suppress cardiac hypertrophy by downregulating several hypertrophic genes. Similarly, miR-101, miR-133, miR-9, miR-98, miR-26, and miR-378 have all been shown to directly and indirectly target anti-hypertrophic cellular pathways to suppress cardiac hypertrophy. Yet other microRNAs, like miR-155, miR-199b, miR-19a, miR-208a/b, and miR-350, have been shown to produce pro-hypertrophic effects. The breadth of microRNAs involved in cardiac hypertrophy illustrates the diverse functions of microRNAs.

MicroRNAs have also shown involvement in cardiac fibrosis. Cardiac fibrosis relates to the accumulation of collagens and other proteins that can lead to heart attack and heart failure. It has been shown that the miR-29 family consisting of miR-29a, miR-29b, and miR-29c, are involved in promoting fibrosis in cardiac fibrosis. This is particularly interesting, as miR-29b is also involved in cancer. However, miR-29b plays a positive role in suppressing cancer
progression. MiR-29b’s ability to promote and suppress different diseases illustrates the multifaceted nature of microRNAs. On the other hand, let-7, miR-26a, and miR-101, and miR-101a have been found to downregulate cardiac fibrosis. Again, it is interesting to note that let-7 has the ability to suppress cardiac fibrosis and tumor progression. Thus, microRNAs like let-7 that serve positive roles across multiple disease systems could be evaluated for therapeutic use.

Lastly, microRNAs have shown involvement in the condition of atherosclerosis. Atherosclerosis refers to the buildup of plaque in blood vessels that can result in a loss of blood flow to the heart. This can lead to myocardial infarctions or heart attacks. The research surrounding microRNAs’ involvement in atherosclerosis is fairly preliminary, but several studies have shown the upregulation of microRNAs relating to the formation of atherosclerosis. While more research needs to be done, these preliminary results suggest that microRNAs could serve as potential biomarkers for the development of atherosclerosis.

The current data surrounding the involvement of microRNAs in cardiovascular conditions provides a new level of depth to microRNAs’ functions. As with cancer, one can see that a continued pattern with microRNAs relates to their ability to both promote and suppress disease. However, the research regarding microRNAs and cardiovascular disease provides a new layer to the microRNA story by demonstrating that microRNAs can be beneficial within one disease system and detrimental within another. Furthermore, cardiovascular disease research surrounding microRNAs illustrates the potential for these molecules to act as biomarkers for disease.
MicroRNAs have shown involvement in a variety of autoimmune disorders. Autoimmune disorders are conditions where the innate and/or adaptive immune system acts aberrantly and attacks healthy tissue and cells. The number of microRNAs that have shown to be involved in autoimmune conditions is extensive, since microRNAs are deeply intertwined with the function of the innate and adaptive immune systems. Namely, microRNAs have shown involvement in systemic lupus erythematosus, primary Sjögren’s syndrome, systemic sclerosis, multiple sclerosis, and rheumatoid arthritis. As seen with cancer and cardiovascular disease, the microRNAs involved in these autoimmune disorders can both aggravate and suppress disease progression. However, one interesting quality of microRNAs involved in autoimmune disorders is that there are certain microRNAs that show involvement across a wide range of autoimmune disorders. More specifically, it appears that miR-155 and miR-146a are involved in all of the autoimmune disorders mentioned and more. While miR-155 and miR-146a are expressed differentially within different autoimmune disorders (ie: decreased expression levels in certain disorders and increased expression levels in others), this consistent involvement of certain microRNAs within many autoimmune disorders is not observed for cancer of cardiovascular disease. Since miR-155 and miR-146a are seen in a wide range of autoimmune disorders, they also present as good disease biomarkers to evaluate autoimmune disorders in a clinical setting. Additionally, it is interesting to note that miR-155 is also involved in cancer and cardiovascular disease, while miR-146a is also involved in cancer. Once again, this demonstrates how certain microRNAs may be good therapeutic targets or biomarkers for multiple diseases.

Ultimately, the current research surrounding the involvement of microRNAs in autoimmune disorders comes together to show that particular microRNAs can be involved in
many subsets of a disease system. As seen with cancer and cardiovascular disease, one can see that a continued pattern with microRNAs relates to their ability to both promote and suppress disease systems. Furthermore, autoimmune disorder research surrounding microRNAs illustrates the potential for these molecules to act as collective biomarkers for autoimmune disorders and other diseases.

2.5: Can Exogenous Dietary MicroRNAs Regulate Mammalian Gene Expression?

The prior sections have demonstrated that endogenously produced mammalian microRNAs can have a tremendous, diverse, and multifaceted impact on disease progression. It is extremely valuable to understand how endogenous microRNAs function and how they can affect many aspects of mammalian and human health. Even one endogenously produced microRNA can have multiple important functions.

However, as previously mentioned, humans are not the only organisms that produce endogenous microRNAs. Endogenous microRNAs are also produced by other mammals and plants – which mammals, including humans, often consume as food. Therefore, this begs the question – can mammals assimilate exogenous microRNAs from food and can these assimilated microRNAs affect gene function? Indeed, current research indicates that this may be possible. This revelation brings another layer of complexity to the relationship between microRNAs and health. Not only can organisms be affected by their own microRNAs, but they can also affected by the microRNAs coming from the food that they consume. The remainder of this section will thoughtfully evaluate the current research surrounding the ability of exogenous dietary microRNAs to assimilate into mammalian sera and affect gene function.
In a paper published in 2014, researchers evaluated the ability of exogenous milk microRNAs, namely miR-29b and miR-200c, to assimilate into human sera in significant amounts. MiR-29b and miR-200c are also endogenously produced by humans, where they positively regulate the gene RUNX2 and negatively regulate the gene ZEB1. The regulatory roles of miR-29b and miR-200c play an important role in the metastasis of cancer. Based on the known functions of miR-29b and miR-200c, researchers knew that these two microRNAs had gene targets in humans. Therefore, in addition to examining if exogenous milk miR-29b and miR-200c could assimilate into human sera, researchers also examined whether or not these exogenous microRNAs had the ability to affect their normal human gene targets. In a human feeding study conducted within this paper, it was found that both miR-29b and miR-200c increased significantly in the sera of humans consuming nutritionally-relevant doses of milk in comparison to a control. Peripheral blood mononuclear cells (PBMCs) were extracted from the members of the human feeding study after milk consumption and analyzed for microRNA levels and gene expression. While miR-200c was increased significantly in these PBMCs, miR-29b did not increase significantly. The PBMCs were also examined for expression of RUNX2 and ZEB1. While researchers found a significant increase in RUNX2 expression, they did not find a significant decrease in ZEB1 expression. Thus, while researchers in this study concluded that exogenous milk microRNAs significantly increased in human sera and affected gene expression, the raw data shows that this is not necessarily the case. The results of this study lead into the problems with this study as a whole. Firstly, the human feeding portion of this study was only conducted in five individuals, which is not a large enough sample size to draw conclusions from. Furthermore, researchers conducted mostly in vitro analysis and failed to examine whether or not these exogenous microRNAs could actually survive the environment of the human digestive
system, including the mouth, small intestine, and large intestine. In addition, the researchers did not clearly show that exogenous microRNAs are forming miRISC complexes and binding the proposed target transcripts. Consequently, the current research surrounding the ability of exogenous dietary milk microRNAs to regulate human gene expression still has many questions that need to be answered. At this point, it is unclear whether or not exogenous microRNAs from milk can have a meaningful impact on human gene expression.

While milk and animal-based food microRNAs are identical or highly similar to mammalian microRNAs, plant food microRNAs are not as analogous to human microRNAs as animal food microRNAs. Therefore, the human/mammalian mRNA targets of plant microRNAs are not as readily apparent. However, a study published in 2012 showed the remarkable phenomena of cross-kingdom regulation, or the ability of exogenous plant microRNAs consumed from food to alter human gene expression. More specifically, this study used a human feeding experiment to show that individuals that consumed a diet high in rice showed increased serum levels of miR-168a, a plant microRNA found abundantly in rice. The researchers then evaluated the ability of the microRNAs to survive in the human and murine GI tract, and they found that miR-168a was able to survive in the GI tract in both models. Furthermore, this study determined that miR-168a could downregulate gene expression for the human gene LDLRAP1, which is responsible for regulating levels of LDL in the bloodstream. Researchers accomplished this by conducting a mice-feeding study where mice were either fed a diet high in rice or a control chow diet. Researchers found that mice fed a rice-high diet significantly downregulated expression of LDLRAP1. When an anti-miR-168a antibody was administered to these mice, the effect on LDLRAP1 was reversed. Lastly, the researchers showed that miR-168a formed the canonical miRISC complex necessary for microRNAs to regulate gene expression. This 2012
study was thorough and covered many holes seen in the aforementioned study evaluating the ability of exogenous milk microRNAs to regulate human gene expression. Unfortunately, the results of this study remain controversial and many have failed to replicate the results. In particular, current research still disputes whether or not exogenous dietary microRNAs can actually survive the environment of the mammalian digestive system and be absorbed into mammalian sera. There is clearly a link between plant microRNAs and human gene expression, and multiple studies have proposed plausible human targets for plant microRNAs. However, the actual evidence surrounding the ability of plant microRNAs to regulate human gene expression via cross-kingdom regulation remains sparse.

The results of these studies come together to show that mammalian gene expression may be affected by exogenous microRNAs acquired through diet. Furthermore, cross-kingdom regulation, where exogenous plant microRNAs modulate mammalian gene expression, may be possible. However, though regulation of human gene expression by exogenous, dietary microRNAs may be possible, more research needs to be done to establish a true connection between exogenous dietary microRNAs and human gene expression.

2.6: The Value of Studying Exogenous Dietary Plant microRNAs with Respect to Human Health

Current research has shown that exogenous plant microRNAs are able to assimilate into mammalian sera and modulate gene expression. This information shows how plant microRNAs can affect mammalian gene expression. However, it is crucial to establish exactly why it is valuable to evaluate exogenous plant microRNAs with respect to human health. Simply put, studying microRNAs in plants with respect to human health is valuable due to the relevance of a
plant-based diet in many health conditions. One thing doctors recommend with many health conditions, including cancer and cardiovascular disease, is switching to or maintaining a healthy diet with a substantial portion of fruits and vegetables. It is clear that eating a diet filled with more fruits and vegetables can be preventative and/or therapeutic in many health conditions. However, medical professionals have yet to understand exactly why. For years, scientists, medical professionals, and the general public have asserted that bioactive compounds like beta carotene, retinol, fiber, Vitamin E, and more are responsible for plants’ beneficial effects in certain health conditions. However, recent studies have shown that this is not the case. A prospective cohort study evaluating the effect of beta carotene and retinol on lung cancer showed that the two compounds had no benefit in lung cancer and showed evidence of having a harmful effect. Another study evaluating the protective effect of Vitamin E in prostate cancer showed that Vitamin E did not have a protective effect, as expected, but actually increased the risk for prostate cancer. In another study evaluating the relationship between fiber and colorectal cancer in women, researchers found that fiber did not improve colorectal cancer prognosis as expected. These results come together to show that individual antioxidants and bioactive compounds may not be as separately responsible for the benefits of plant foods as previously thought. It suggests that these compounds could work together to afford beneficial effects, and that there may be other beneficial bioactive compounds working within plants, like microRNAs. This is why it is valuable to study beyond the scope of what is known and evaluate exogenous microRNAs from plants as a potential bioactive compounds that can have important health effects.
2.7: The Effects of Processing on MicroRNA Content in Food

When studying bioactive compounds within plants, it is important to consider how processing will affect the availability of those compounds. This obviously also applies to studying exogenous microRNAs. Often, the most consumed varieties of a food are the processed varieties. While no work has been done evaluating how processing affects microRNA levels in plants specifically, some research has been done on how processing affects microRNA content in other foods. In particular, one study evaluated the effects of milk processing on levels of miR-29b and miR-200c. Mir-29b and miR-200c are important disease-involved microRNAs, as previously discussed. This study found that pasteurization and homogenization decreased miR-29b and miR-200c by 63% and 67%, respectively, in comparison to unprocessed milk. Heated milk showed a 40% decrease in miR-29b, but not miR-200c. While these results cannot be directly extrapolated to how processing affects microRNA content in plants, it provides an idea of how different levels of processing can lower levels of microRNAs. Overall, it appears that harsher processing seems to more intensely decrease microRNA content within foods.

2.8: Summary and Application

Ultimately, endogenous human microRNAs can have an enormous, diverse impact on human health. Since microRNAs are found in other organisms, namely plants, it is logical to examine if exogenous plant microRNAs obtained through diet can affect human gene expression in ways similar to endogenous human microRNAs. Studying exogenous plant microRNAs is especially relevant to disease progression, since a recommended course of action for many diagnoses is to adopt a diet rich in plant foods. More specifically, since there is still a significant
amount of confusion over what makes plant foods beneficial to health and disease prognosis, it is helpful to evaluate microRNAs as potential bioactive compounds. MiR-168a is a plant microRNA that has been shown to assimilate into mammalian sera and modulate gene expression. Therefore, this project sought to evaluate levels of miR-168a in processed potatoes and the sera of pigs that had been fed those processed potatoes. Since processing is a huge facet of the food industry and how people consume food, it is essential to examine how processing affects the levels of a bioactive compound, like microRNAs. The results of a study such as this one could be used to advise the food industry on how to process potatoes so as to preserve beneficial microRNAs.
3.1: Preparation of Potato and Serum Samples

Raw, baked, and chipped White Atlantic and Purple Majesty potatoes were obtained from the Pennsylvania State University Food & Gut Health Laboratory. White Atlantic and Purple Majesty were grown at the San Luis Valley Research Center, Colorado State University, Center, CO, USA in 2012. Potatoes of each cultivar were then processed via baking and chipping. All potatoes were washed with room temperature water before processing. Raw samples of potato from each cultivar were diced with skin into pieces weighing 7 ± 1g and stored at -20°C until analysis. Medium sized potatoes (6 to 7 oz) each wrapped in food-grade aluminum foil and pierced with a knife approximately 1.5 cm deep at 3 cm intervals were baked for 1 hour in a conventional oven preheated to 204°C, then allowed to cool for 30 minutes at room temperature. After cooling, potatoes were diced with skin and stored at -20°C until analysis. Chip slices were made using an industrial slicer (Ditto Dean Food Prep TR23; Rocklin, CA) with a C-2 blade. Raw chips were washed under running warm water for approximately 1 minute to remove any water-soluble sugars present on the surface and placed in strainer trays for 5 minutes to remove excess water. Chips were fried in a 5-liter capacity fryer from APW Wyott EF-30-208-2 (Allen, TX) with Sam’s Club Bakers & Chefs Clear Frying Oil (Bentonville, AR). Chip slices were fried at 185°C for 2 minutes and then stored at -20°C until analysis.
Serum samples were obtained from a previous study where pigs were fed with raw, baked, and chipped White Atlantic and Purple Majesty potatoes. Animals (n = 64) were divided into eight groups and provided with one of the following diets: a standard control diet (C1, ~5 % fat), a HCD control (C2, 17 % dry fat and ~3-5 % endogenous fat) or 6 different diets with the HCD supplemented with 10 % of either purple or white-fleshed potato, raw, baked or chips. Purple and white-fleshed potatoes were grown at Black Gold Farms (Pearsall, TX). Processing of potatoes i.e. baking and chipping was done at Worldwide Foods (Burley, ID) and Kettle Foods (Salem, OR) respectively. Additionally, raw and baked potatoes were freeze-dried at Van Drunen Farms (Momence, IL) prior to incorporation in the diet. White corn and fat were used as a major energy source, and soybean meal was the major protein source. Ratios between corn and soybean meal were adjusted to match energy and protein contents among diets. White corn was used to prevent carotenoids in the yellow corn from affecting the study. A proximate analysis was conducted on the potato material IEH-Warren Laboratory (Greeley, CO) before incorporation into diet to ensure that macronutrient composition was similar across the groups. Composition of all of the diets is presented in Figure 1. Pigs consumed the experimental diets for 13 weeks; the feed and drinking water were provided ad libitum.
## Figure 1: Diet Composition for Prevention Study

<table>
<thead>
<tr>
<th>Ingredients (%)</th>
<th>Standard Control</th>
<th>High-Calorie Control</th>
<th>Purple Raw</th>
<th>White Raw</th>
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**Composition (% unless mentioned)**

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<th>Methionine (%)</th>
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C1, Standard control, C2, High-calorie diet control, letters P and W indicate purple- and white-fleshed potato diets, R, B & C indicate processing treatments raw, baked and chips, respectively at 10% supplementation.
3.2: Total RNA Extraction

Raw, baked, and chipped White Atlantic and Purple Majesty potato samples were powdered using liquid nitrogen. Total RNA was extracted from these six groups of potatoes in duplicates using the Life Technologies mirVana miRNA Isolation Kit, with phenol. Total RNA was extracted from both potato and serum samples using the Qiagen miRNEasy Serum/Plasma Kit.

3.3: qRT-PCR

Reverse Transcription for potato and serum extracts to cDNA was performed using the Qiagen miScript II RT Kit. Mature miRNAs were polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers and miScript HiSpec Buffer. The oligo-dT primers have a 3’ degenerate anchor and a universal tag sequence on the 5’ end, allowing amplification of mature miRNA in real-time qPCR. The cDNA produced through reverse transcription was subjected to qPCR using a Qiagen Custom Primer for miR168a for all samples (Mature Sequence: 5’UCGCUUGGUGCAGGUCGGGAA) using the Qiagen miScript SYBR Green PCR Kit. For serum extracts, an additional qPCR for miR-39a was performed for normalization using a Qiagen Custom Primer for miR-39a (Mature Sequence: 5’UCACCGGGUGUAAAUCAGCUUG).
3.4: Standard Curve Preparation

A standard curve was generated to convert Cq values from PCR into concrete concentrations. The standard curve was prepared by dilution of the miReasy Serum/Plasma Spike-In Control (C. elegans miR-39 mimic). Each dilution was analyzed via qPCR using the Qiagen miScript SYBR Green PCR Kit to obtain Cq values for each dilution. Following qPCR analysis, a standard curve plotting the log[copy number] against the mean Cq value for each sample.
Chapter 4
Results

4.1: Processing Drastically Decreases miR-168a Content In White Atlantic and Purple Majesty Potato Products

![Bar Chart](image)

*Figure 2: Relative Levels of miR-168a in White Atlantic and Purple Majesty Potato Products*

[* = a statistically significant result (p-value < 0.05) in comparison to the ‘Raw’ control]*

Firstly, this study sought to understand the effects of processing, namely baking and chipping, on the miR-168a content within Purple Majesty and White Atlantic potato products. To evaluate this question, the miR-168a levels within raw, baked, and chipped Purple Majesty and White Atlantic Potatoes were examined. In comparison to a raw control, it was found that baking and chipping resulted in a drastic, significant decrease in miR-168a content within both Purple Majesty and White Atlantic potato products. There did not appear to be a significant difference
between baking and chipping in terms of which processing decreased miR-168a content more. Thus, it appeared that both types of processing comparably decreased miR-168a content within both examined varieties of potatoes in comparison to a raw control. These results are visualized in Figure 2, above.

Interestingly, raw Purple Majesty samples displayed much higher levels of miR-168a than raw White Atlantic samples. However, after processing, both baked and chipped White Atlantic and Purple Majesty potato products showed comparable miR-168a content. Thus, though there were greater levels of miR-168a within raw Purple Majesty samples initially, this difference did not seem to carry over after processing. Between White Atlantic and Purple Majesty samples, there did not appear to be a variety that better retained miR-168a after processing. These results are also visualized in Figure 2, above.

As a whole, these results come together to indicate that processing through baking and chipping drastically and significantly decreased miR-168a content in both White Atlantic and Purple Majesty potato products.
Figure 3: Relative Levels of miR-168a in Serum of Pigs Fed White Atlantic and Purple Majesty Potato Products

[* = Statistically significant result (p value < 0.05) in comparison to the “Corn” Diet]

4.2: Plant MicroRNAs Are Absorbed from White Atlantic Potato Products, Purple Majesty Potato Products, and Corn into Mammalian Serum

Following analysis of White Atlantic and Purple Majesty potato products for miR-168a content, the levels of miR-168a were examined in the serum of pigs that had been fed diets consisting of raw corn alone and raw corn in combination with raw, baked, and chipped White Atlantic and Purple Majesty potatoes. This pig feeding study or prevention study was performed prior to the start of this study. The pigs fed a combination of raw corn and potato products were fed much less raw corn than pigs fed a diet of corn alone. The diet compositions for the pigs in the prevention study are more clearly presented in Figure 1 in Chapter 3. In the current study, the serum samples from this pig feeding study (also referred to as a prevention study) were analyzed
for miR-168a content, and the miR-168a content of these pigs’ sera is displayed above in Figure 3.

Based on the results shown in Figure 3, it appeared that there was miR-168a present in the serum of pigs fed a diet of corn alone. This indicated that miR-168a was absorbed from corn alone into mammalian sera. In addition, as shown in Figure 3, there was also miR-168a present in the serum of pigs fed a combination of raw corn and Purple Majesty or White Atlantic potato products. Since the pigs fed a combination of raw corn and potato products were fed a much lower amount of corn than those fed corn only, it is likely that a portion of the miR-168a visualized in these pigs’ sera was from potato products. These results come together to demonstrate that plant miR-168a from raw corn, Purple Majesty potato products, and White Atlantic potato products is absorbed via dietary means into mammalian serum.

4.3: Processing of White Atlantic and Purple Majesty Potatoes Decreases the Availability of miR-168a for Absorption into Mammalian Serum

Upon establishing that plant miR-168a was absorbed from dietary raw corn and potato products into mammalian serum, this study next sought to evaluate how processing of Purple Majesty and White Atlantic potato products affected the absorption of miR-168a into mammalian serum.

Firstly, the levels of miR-168a in the serum of pigs fed a combination of White Atlantic potato products and raw corn were evaluated (Figure 3). When pigs were fed a combination of processed (baked or chipped) White Atlantic potato products with raw corn, it significantly decreased the levels of serum miR-168a in comparison to those fed a diet of raw White Atlantic potato products and raw corn.
Secondly, the levels of miR-168a in the serum of pigs fed a combination of Purple Majesty potato products and raw corn were evaluated (Figure 3). When pigs were fed a combination of baked Purple Majesty potato products and raw corn, it significantly decreased the levels of serum miR-168a in comparison to those fed a diet of raw Purple Majesty potato products and raw corn.

Thus, as displayed in Figure 3, these results come together to demonstrate that processing of White Atlantic and Purple Majesty potato products decreases the availability of miR-168a for dietary absorption into mammalian sera.

4.4: Absorption of miR-168a into Mammalian Serum from White Atlantic and Purple Majesty Potatoes is Processing-Type Dependent and Variety Dependent

After determining that processing of White Atlantic and Purple majesty potato products decreased the availability of miR-168a for dietary absorption into mammalian sera, the effects of processing-type and potato variety on miR-168a absorption into mammalian sera were examined. As seen in Figure 3, across pigs fed some combination of Purple Majesty potato products or White Atlantic potato products, serum miR-168a was significantly higher in those pigs fed chipped potato varieties as opposed to baked varieties. This suggests that absorption of miR-168a into mammalian serum from dietary potato products is dependent on processing, and that chipping allows for better miR-168a absorption into mammalian serum.

Additionally, as visualized in Figure 3, it appeared that serum levels of miR-168a were much lower in pigs fed some combination of Purple Majesty potato products than pigs fed some combination of White Atlantic potato products. These results come together to suggest that
absorption of dietary miR-168a into mammalian sera from White Atlantic and Purple Majesty potato products is dependent on the variety of potato.
MiRNAs are quickly becoming a domain of interest in the scientific community with respect to their ability to act as bioactive compounds in plant-based foods. This study sought to expand upon the current research surrounding miRNAs’ role as a bioactive compound.

Firstly, the effects of processing, namely baking and chipping, on levels of miR-168a within White Atlantic and Purple Majesty potatoes was examined. It was found the both types of processing fairly comparably and significantly reduced miR-168a content within both varieties of potatoes in comparison to a raw control. These results indicate that conventional processing methods, such as baking and frying, are not very efficient at retaining microRNAs. As the field of microRNAs grows and the importance of miRNAs as bioactive compounds is established, it will become necessary to evaluate new, novel food processing methods to better retain microRNAs. One such processing method that could be evaluated is vacuum frying. As a whole, the results of this study indicate that conventional methods are not sufficient to retain miRNAs and that research on new processing methods needs to be carried out.

Next, this study observed that exogenous dietary plant miR-168a from corn, White Atlantic potato products, and Purple Majesty potato products, were absorbed into mammalian sera. This result is particularly notable, as the ability of exogenous plant microRNAs to assimilate into mammalian sera has been disputed in the past. Thus, this study presents new evidence that exogenous microRNAs can be absorbed into mammalian serum through dietary means.

This study then expanded upon prior research by evaluating how food processing in White Atlantic and Purple Majesty potato products may affect the availability of miR-168a for
absorption into mammalian sera via dietary means. The results indicate that food processing, namely baking and chipping, reduced the availability of miR-168a for absorption into mammalian sera. As previously noted, this study also observed a reduction in miR-168a levels when White Atlantic and Purple majesty potato products were processed. Thus, the reduced availability of microRNAs for serum absorption after food processing is logical.

Lastly, this study found that the variety of potato and processing-type of potato affected the availability of miR-168a for absorption into mammalian sera. Specifically, Purple Majesty potato products seemed to render miR-168a less available for absorption into mammalian sera than White Atlantic potato products. This result is valuable and suggests that more cultivars should be evaluated to understand how variety may affect the availability of miR-168a. In addition, this study also observed that chipping better retained miR-168a for absorption into mammalian serum than baking. This is interesting, as chipping of both white and color-flesh potatoes has been shown to lead to a drastic reduction in health-related compounds such as phenols, anthocyanins, Vitamin C, and other antioxidants. It is possible that the increased availability of miR-168a for absorption in chipped potatoes has to do with the microstructure of potatoes after processing. Research has shown that chipping drastically reduces water content within potatoes. Many enzymes of the GI tract are water active and require water to process food. Thus, perhaps the increased availability of miR-168a within chipped potatoes is due to the inability of water-soluble enzymes in the GI tract to access microRNAs within water-lacking chips. This is just one possible reason for the increase availability of miR-168a from chipped potatoes for serum absorption. Overall, this result suggests that more research needs to be done to evaluate how the GI tract may affect the availability of dietary microRNAs for absorption into mammalian sera.
Chapter 6
Conclusions and Future Directions

6.1: Conclusions

In conclusion, it appears that processing of potatoes (baking and chipping) drastically decreased miR-168a levels in comparison to a raw control for both White Atlantic and Purple Majesty potato products. Secondly, it appears that exogenous dietary miR-168a (from both White Atlantic and Purple Majesty samples) is absorbed into the sera of pigs. Specifically, the results of this study confirm that exogenous miR-168a can be absorbed from corn, White Atlantic potato products, and Purple Majesty potato products into the serum of pigs. Furthermore, processing of potatoes may affect how well this exogenous miR-168a is absorbed into the sera of pigs. Specifically, it seems that chipping of potatoes may render miR-168a more available for absorption than baking. Lastly, there appears to be a variety-dependent difference in the absorption of exogenous, dietary miR-168a. Specifically, it seems that miR-168a is better absorbed into the serum of pigs from White Atlantic potato products than Purple Majesty potato products.

6.2: Future Directions

This study has many potential future directions. Firstly, both the potato and pig feeding studies could be expanded to include other varieties of potatoes, other types of potato processing, and evaluation of other relevant miRNAs. However, the expansion of this study should not be restricted to potato cultivar or processing type. In fact, the entire farm-to-fork spectrum needs to be examined. Future studies should examine how things such as operations and storage affect
levels of different microRNAs within not only different varieties of potatoes, but all miRNA containing foods. In addition to expanding the current research to include the entirety of the farm-to-fork spectrum, more consideration needs to be given to how the mammalian GI tract and physiology affects the uptake and assimilation of miRNAs from food. Specifically, more research needs to be done to not only understand how this physiology affects uptake of miRNA from food, but how the combination of food processing (ie: farm-to-fork spectrum) and mammalian physiology affects the uptake of dietary miRNAs. Lastly, more needs to be done to understand if miRNAs assimilated into mammalian serum have the ability to meaningfully affect mammalian gene function.
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