Glycemic and insulinemic responses to resistant starch type 4

by

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B.S., University of the Incarnate Word, 2014
M.S., Texas A&M University, 2017

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Department of Food, Nutrition, Dietetics, and Health
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Abstract

The overall aim of this dissertation was to determine the effects of resistant starch type 4 (RS4) on postprandial glycemic and insulinemic responses, and to determine whether there were differences between United States Food and Drug Administration (FDA) approved testing methods (add-on or substitution, with matched digestible carbohydrate amounts) and non-FDA approved testing methods (substitution without matched digestible carbohydrate amounts).

The first study (Chapter 2) examined the impact of RS4 consumption on glycemic and insulinemic responses using the FDA approved add-on method, a method of adding the fiber on top of the control treatment, and the non-FDA approved substitution method, a substitution method used for manufacturing products available to consumers. We used a randomized controlled crossover design to compare postprandial glycemic and insulinemic responses following consumption of RS4 crackers and native wheat starch (NWS) crackers. The results indicated that insulin incremental area under the curve (iAUC) was significantly lower (~87%) following consumption of RS4 crackers compared with NWS crackers manufactured using the non-FDA approved substitution method. However, no differences were observed in glycemic or insulinemic responses following consumption of RS4 or NWS crackers manufactured using the FDA approved add-on method (*p*s>0.05). These findings suggest that the FDA approved add-on method and non-FDA approved substitution method should be further investigated to elucidate the importance of the differences between the way ingredients are tested versus how they are packaged, purchased, and used by consumers.

Therefore, the second study (Chapter 3) utilized the FDA approved substitution method where RS4 was substituted in place of a carbohydrate containing ingredient (puffed wheat) in the control treatment. Using a randomized controlled crossover design, we investigated the

postprandial glycemic and insulinemic responses following consumption of RS4 nutrition bars and puffed wheat bars (PWB) across two doses of digestible carbohydrate, one more typical of FDA testing, and one more typical of how products are manufactured, packaged, and purchased by consumers. This study revealed that glucose and insulin iAUC were significantly lower in the 30g digestible carbohydrate conditions compared to the 50g digestible carbohydrate conditions (p<0.05) and were also significantly lower in RS4 conditions compared with PWB and dextrose conditions (ps<0.05). Results for the RS4 nutrition bars indicated a mean reduction of ~50% for glucose iAUC and a mean reduction of ~46% for insulin iAUC compared with the PWB and dextrose conditions. These findings suggest that RS4 produces beneficial glycemic and insulinemic responses, regardless of the dose of digestible carbohydrate.

To further investigate the effects of RS4 on glycemic and insulinemic responses using the FDA approved and non-FDA approved testing methods, Chapter 4 reports the results of a systematic review and meta-analysis of human randomized clinical trials investigating the effects of RS4 on postprandial glucose and insulin iAUC. The meta-analysis indicated that RS4 significantly reduces postprandial glucose and insulin iAUC compared to control conditions. Further, a subgroup analysis confirmed that glucose iAUC was significantly lower compared with control condition across both FDA approved and non-FDA approved testing methods. However, in studies that used FDA approved substitution methods, insulin iAUC was not different between RS4 and control conditions, whereas there were significant reductions in insulin iAUC following RS4 consumption observed in studies using the non-FDA approved substitution method. These collective findings indicate an overall reduction in postprandial glycemic and insulinemic responses following RS4 consumption when compared to control conditions, consistent with the current literature. However, the beneficial responses are more

consistent when utilizing digestible carbohydrate amounts and testing methods typical of products that can be purchased by consumers.

Overall, the studies included in this dissertation suggest beneficial metabolic effects following RS4 consumption and indicate a need for further investigation of the use of RS4 in product development. Substitution of RS4 in carbohydrate containing products may be a potentially feasible approach for helping to mitigate the increasing burden of non-communicable chronic diseases, metabolic diseases in particular.

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To further investigate the effects of RS4 on glycemic and insulinemic responses using the FDA approved and non-FDA approved testing methods, Chapter 4 reports the results of a systematic review and meta-analysis of human randomized clinical trials investigating the effects of RS4 on postprandial glucose and insulin iAUC. The meta-analysis indicated that RS4 significantly reduces postprandial glucose and insulin iAUC compared to control conditions. Further, a subgroup analysis confirmed that glucose iAUC was significantly lower compared with control condition across both FDA approved and non-FDA approved testing methods. However, in studies that used FDA approved substitution methods, insulin iAUC was not different between RS4 and control conditions, whereas there were significant reductions in insulin iAUC following RS4 consumption observed in studies using the non-FDA approved substitution method. These collective findings indicate an overall reduction in postprandial glycemic and insulinemic responses following RS4 consumption when compared to control conditions, consistent with the current literature. However, the beneficial responses are more

consistent when utilizing digestible carbohydrate amounts and testing methods typical of products that can be purchased by consumers.

Overall, the studies included in this dissertation suggest beneficial metabolic effects following RS4 consumption and indicate a need for further investigation of the use of RS4 in product development. Substitution of RS4 in carbohydrate containing products may be a potentially feasible approach for helping to mitigate the increasing burden of non-communicable chronic diseases, metabolic diseases in particular.

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Chapter 1 - Introduction

Prevalent non-communicable chronic health conditions, such as cardiovascular diseases and diabetes mellitus, have serious impacts on healthcare, the economy, and an individual's daily lifestyle (1, 2). Research has established dietary intake as a modifiable lifestyle factor that can affect non-communicable chronic diseases (3). Thus, feasible dietary interventions are of interest in order to reduce the burden of non-communicable chronic diseases. Recently, dietary fiber has been identified as a specific dietary component that could play a role in reducing the prevalence of chronic diseases (4). Research has identified numerous health benefits following acute and chronic fiber consumption (5, 6). These health benefits include, but are not limited to, reductions in postprandial glucose and insulin levels, cholesterol levels, blood pressure, and body weight (5). Despite the positive benefits of fiber, a mere 10% of Americans report consumption at the recommended dietary intake (7). This widespread failure to meet guidelines suggests an opportunity for dietary improvement that may reduce the individual and societal burdens of non-communicable chronic diseases.

Resistant Starch

Resistant starches are specific types of starches that have recently been classified as fibers by the Food and Drug Administration (FDA) and are naturally occurring in some foods and may also be manufactured and substituted for carbohydrate ingredients in products (8). This sort of approach may play an important role in increasing dietary fiber intake. Resistant starch has been shown to improve metabolic health outcomes associated with diabetes mellitus, cardiovascular diseases (CVD), and metabolic syndrome (9, 10).

Resistant starch is a type of starch that resists digestion by escaping the small intestine and entering the large intestine for fermentation, thus acting as a fiber (11). There are five types

of resistant starches that have been recognized and are classified according to the physical and chemical characteristics of the starch (12). Resistant starch type 1 is naturally occurring starch granules encased inside plant cell walls, and type 2 is natural starch comprised of one-half or more amylose content (13). Resistant starch type 3 is isolated starch, or a starchy food, that has undergone hydrothermal treatment followed by cooling, which results in a strong re-association of starch molecules that resist digestion (13). Resistant starch type 4 (RS4) is a chemically modified starch, producing cross-linking that inherently resists digestion, primarily derived from wheat, tapioca, or potato (13). Lastly, resistant starch type 5 is an amylose-lipid complex that is commonly found in cereal starch granules and in processed starch (13). There is a large body of research on resistant starch types 1–3 demonstrating both acute and long-term beneficial effects on human health (14). However, research investigating the use of RS4 and RS5 on human health is limited. Emerging evidence suggests similar health benefits following RS4 consumption as compared with resistant starch types 1–3 (15-25).

Resistant starch type 4 was first developed in 1999 and recently received approval (March 2019) following the new FDA definition of fiber announced in 2016 (26). Prior to 2016, the FDA did not define the term dietary fiber but maintained a list of approved fibers according to the previous regulations. In addition to the new definition, the FDA also included a list of seven non-digestible carbohydrates that the FDA determined had physiological effects beneficial to human health. Following the new FDA guidance, RS4 was not included among the seven fibers determined beneficial to human health. Recently, research has confirmed beneficial effects on human health in the postprandial period following acute consumption of RS4 when substituted for carbohydrate containing ingredients in food, although there is limited evidence of the stated beneficial health outcomes utilizing an FDA approved testing method (15, 17, 27).

Research on RS4 has been conducted using both FDA approved and non-FDA approved methods, leaving the true effects of RS4 consumption uncertain.

Fiber Food Label Classification

The FDA recently defined fiber as "non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units), and lignin that are intrinsic and intact in plants; isolated or synthetic non-digestible carbohydrates (with 3 or more monomeric units) determined by FDA to have physiological effects that are beneficial to human health" (26). The FDA includes benefits such as reductions in blood glucose, cholesterol levels, blood pressure, and energy intake (via increases in satiety) as acceptable for meeting the requirements for fiber classification. In addition, increases in the frequency of bowel movements and mineral absorption in the intestinal tract are also classified as physiological effects beneficial to human health (26).

Research suggests that large excursions in postprandial glycemic and insulinemic responses are associated with increased risk for cardiovascular diseases and type 2 diabetes mellitus (28, 29). Thus, smaller increases in glycemic and insulinemic responses in the postprandial period may be beneficial for human health. Glycemic and insulinemic assessments are typically conducted following an oral glucose tolerance test protocol, where participants go to the laboratory following an overnight fast and blood samples are collected at baseline and serially for two hours following consumption of the designated treatment. According to the FDA, attenuations in glucose and/or insulin levels are physiological effects that indicate benefits for human health (26). As such, glucose and insulin levels can be measured in a postprandial period to assess a potentially beneficial response to human health in order to obtain fiber food label classification. As stated in the FDA's Guidance for Industry, for beneficial physiological effects on human health, the postprandial measurement for glycemic and insulinemic responses must be

measured for a minimum of two hours (26). Therefore, food manufacturers seeking to obtain fiber classification using glycemic and insulinemic responses must submit evidence from clinical studies that use study designs that include a 2-hour postprandial comparison of control and test treatments matched for digestible carbohydrate.

In order to determine the beneficial effects on human health outlined by the FDA, the FDA requires the use of a specific testing protocol to obtain fiber food label classification (26). The FDA approved testing methods require matching of digestible carbohydrate between test and control treatments, with the only difference between treatments being the inclusion of the fiber ingredient (26). Specifically, digestible carbohydrates are carbohydrates available for digestion in the small intestine that can produce energy for cells (30). By matching for digestible carbohydrate, with matched ingredients across treatments, results from a clinical study can provide evidence on the independent physiological effects of the fiber ingredient (26). Substitution and add-on approaches are two methods accepted by the FDA, as long as treatments are matched for digestible carbohydrate, and ingredients are matched across treatments (26). Thus, the starch component of flour can be substituted in place of intact flour in food products. The two accepted methods are described below.

1. The add-on approach adds the test ingredient on top of the control treatment. For example, the starch ingredient is added on top of the control treatment formula, yielding a control treatment without the starch ingredient and a test treatment formulated with the addition of the starch ingredient.
2. The substitution approach substitutes the test ingredient, either partially or fully, in place of a carbohydrate containing ingredient. For example, the starch ingredient is substituted in place of flour in the control formula, yielding testing

treatments that differ only by the inclusion of starch (test treatment) versus flour (control treatment).

It is important to note that both approaches must maintain a matched amount of digestible carbohydrate between test and control treatments in order to investigate the independent effect of the starch. While matching for digestible carbohydrates is the best way to parse the effects of starch on health, neither of the approved methods represent testing of products that closely resemble products that are manufactured and sold in the marketplace to consumers. Taking this one step further, this means that testing does not represent the products that consumers purchase and consume and therefore physiological responses may not be consistent. Typically, the method to produce products for consumers uses a substitution approach, herein referred to as the marketplace substitution method. The marketplace substitution method substitutes the test ingredient, partially or fully, in place of a carbohydrate containing ingredient without matching for digestible carbohydrate. The marketplace substitution approach typically reduces the carbohydrate content of a product, and as a result, the total caloric content of the product. Both reduced carbohydrate and calorie content may elicit a reduced glycemic response, indicative of a beneficial outcome. However, the reported beneficial response may not be due to the resistant starch per se, but rather the reduction in digestible carbohydrate, that may not require as much insulin to clear glucose from the blood(20). Thus, the independent effect of resistant starch cannot be determined using non-FDA approved substitution methods where digestible carbohydrates are not matched. Therefore, testing methods that may provide more accurate physiological responses to resistant starch consumption are not currently approved by the FDA.

Of note, the current body of evidence includes the use of both the FDA approved testing methods (15, 17, 19, 20, 27) and the non-FDA approved marketplace substitution method (16,

18, 20-25). Therefore, a thorough investigation of the differences between these testing methods is needed to elucidate whether the beneficial effects of RS4 are consistent across testing methods.

Overview

The studies included in this document focus on elucidating the effects of resistant starch type 4 on postprandial glycemic and insulinemic responses using FDA approved and non-FDA approved testing methods. The randomized controlled crossover trial reported in Chapter 2, and published in Current Developments in Nutrition, investigated the postprandial metabolic responses to native wheat starch (NWS) versus resistant starch type 4 (RS4) using digestible carbohydrate-matched portions (FDA approved method) compared to weight-matched portions (non-FDA approved method). Chapter 3 is the manuscript of our randomized controlled crossover trial that investigated whether glycemic and insulinemic responses were different when using an amount of digestible carbohydrate more commonly used in research as compared with a consumer-friendly portion more typical of a product purchased in the marketplace. Lastly, Chapter 4 includes a systematic review and meta-analysis of randomized controlled trials investigating glycemic and insulinemic responses to RS4 consumption. In addition, a sub-analysis is included to determine whether similar effects of RS4 on glycemic and insulinemic responses are observed when comparing studies where the FDA approved testing methods were used versus studies where the non-FDA approved marketplace substitution method were used. Overall, the series of studies included in this dissertation will add to the current body of evidence investigating the potential benefits of RS4 consumption on human health outcomes. Specifically, these studies contribute to the current evidence through an investigation of FDA approved and non-FDA approved testing methods for fiber classification (Chapter 2), a novel comparison of

two doses of RS4 using an FDA approved testing method (Chapter 3), and finally a thorough systematic review and meta-analysis of the current body of evidence for the effects of RS4 consumption on glycemic and insulinemic responses in the postprandial period (Chapter 4). Collectively, the studies included herein elucidate the overall impact of RS4 on glycemic and insulinemic response and indicate a need for further investigation of the use of RS4 in product development.

Chapter 2 - Metabolic Responses to Native Wheat Starch (Midsol™ 50) Versus Resistant Wheat Starch Type 4 (Fibersym® RW): Standard Versus Marketplace Testing Protocols

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Structured Abstract

Background: To investigate the effect of RS on acute glycemic or insulinemic responses, the FDA indicates that control and RS-enriched foods must contain equivalent amounts of digestible carbohydrate. However, RS-containing foods typically contain less digestible carbohydrate per serving than control foods. Thus, controlling for digestible carbohydrate may yield different responses as compared with controlling for serving size.

Objective: To compare the postprandial metabolic responses to native wheat starch (NWS) versus resistant starch type 4 (RS4) using digestible carbohydrate-matched portions compared to weight-matched portions.

Methods: A single-blinded randomized-controlled crossover trial examined glycemic and insulinemic responses over two hours following consumption of four cracker conditions and a dextrose beverage in apparently healthy participants (n=14). Crackers provided 50g of digestible carbohydrate (CHO) using the FDA's meal-intervention protocol, or 35g of CHO by weight for the marketplace substitution method. Crackers differed only by the type of starch additive: NWS (Midsol^{TM} 50) or RS4 (Fibersym$^{®}$ RW). Glucose levels were assessed at baseline, 15, 30, 45, 60, 90, and 120 minutes; insulin levels were measured at baseline, 30, 60, and 120 minutes.

Results: There were no significant differences between 50g digestible CHO cracker conditions for glucose or insulin iAUC. The 35g CHO by weight conditions were not different for glucose iAUC (Mean (95% CI); 35g NWS: 1317 (677, 2169), 35g RS4: 701 (262, 1351), $p>0.05$). However, insulin iAUC was lower following 35g RS4 compared to 35g NWS (35RS4: 92 (10, 259), 35NWS: 697 (397, 1080), $p<0.01$).

Conclusion: In healthy adults, consumption of RS4 crackers decreased postprandial insulin responses compared with NWS crackers when using the marketplace substitution method

compared to the FDA standard testing method, with similar postprandial glucose responses. Comparisons of the FDA standard testing method and the marketplace substitution method should be investigated further to elucidate differential physiological impacts on consumers.

Unstructured Abstract

To investigate the effect of RS on acute glycemic or insulinemic responses, the FDA indicates that control and RS-enriched foods must contain equivalent amounts of digestible carbohydrate. However, RS-containing foods typically contain less digestible carbohydrate per serving than control foods. Thus, controlling for digestible carbohydrate may yield different responses as compared with controlling for serving size. To compare the postprandial metabolic responses to native wheat starch (NWS) versus resistant starch type 4 (RS4) using digestible carbohydrate-matched portions compared to weight-matched portions. A single-blinded randomized-controlled crossover trial examined glycemic and insulinemic responses over two hours following consumption of four cracker conditions and a dextrose beverage in apparently healthy participants (n=14). Crackers provided 50g of digestible carbohydrate (CHO) using the FDA testing method, or 35g of CHO by weight for the marketplace substitution method. Crackers differed only by the type of starch additive: NWS (Midsol^{TM} 50) or RS4 (Fibersym$^{®}$ RW). Glucose levels were assessed at baseline, 15, 30, 45, 60, 90, and 120 minutes; insulin levels were measured at baseline, 30, 60, and 120 minutes. There were no significant differences between 50g digestible CHO cracker conditions for glucose or insulin iAUC. The 35g CHO by weight conditions were not different for glucose iAUC (Mean (95% CI); 35g NWS: 1317 (677, 2169), 35g RS4: 701 (262, 1351), $p>0.05$). However, insulin iAUC was lower following 35g RS4 compared to 35g NWS (35RS4: 92 (10, 259), 35NWS: 697 (397, 1080), $p<0.01$). In healthy adults, consumption of RS4 crackers decreased postprandial insulin responses compared with

NWS crackers when using the marketplace substitution method compared to the FDA standard testing method, with similar postprandial glucose responses. Comparisons of the FDA standard testing method and the marketplace substitution method should be investigated further to elucidate differential physiological impacts on consumers.

Keywords**:** resistant starch type 4, RS4, postprandial glycemia, postprandial insulinemia, metabolic syndrome, healthy adults, fiber, substitution method, FDA standard testing method

Summary

Resistant starch type 4 crackers reduced insulin response despite similar glycemic response to control crackers. Differential insulinemic responses were observed following FDA standard testing methods and marketplace substitution testing methods.

Introduction

Low consumption of dietary fiber, which is principally indigestible carbohydrate, is associated with reduced insulin sensitivity and increased risk for type 2 diabetes mellitus (31). Consumption of dietary fiber has shown promising outcomes associated with the reduction of risk for metabolic disease, including improving metabolic response outcomes, promoting satiety, and for obesity prevention and treatment (14). While numerous studies have shown the beneficial health outcomes associated with dietary fiber consumption, national consumption remains at approximately 50% of the recommended dietary intake (32-34). Resistant starch type 4 (RS4) has recently emerged as a fiber (35) and has shown beneficial health outcomes in the postprandial period following acute consumption, when substituted for flour in food (15, 22-24, 36, 37). However, there is limited evidence to show an effect of RS4 on glycemic and insulinemic responses when the test and control foods are matched for digestible carbohydrate.

The FDA maintains two methods of acceptable protocols to test a fiber ingredient for food-labeling purposes. The test ingredient should either be added on top of all ingredients, or substituted in place of an ingredient, all the while matching for digestible carbohydrates between the test and control foods (26). In contrast to the FDA protocol, the marketplace substitution method simply replaces an ingredient with the fiber. Fiber-fortified foods in the marketplace are not formulated to match the digestible carbohydrate of the original products; rather the fiber ingredients replace digestible carbohydrates. This typically reduces the total caloric and digestible carbohydrate content of a product, while increasing the fiber content.

The FDA standard testing method often increases the total caloric content of a test food or requires large amounts of that food to be consumed during testing sessions. In comparison, using the marketplace substitution method can decrease the total caloric content for a test food

when digestible carbohydrates are not matched. These methods are important for the food industry, as any food that has shown a beneficial health outcome in accordance with FDA regulations can be labeled with a health claim (38). These health claims are tightly regulated by the FDA as a single health claim on a food product has been shown to increase sales substantially (39). Therefore, it is important to compare FDA standard testing protocols to marketplace substitution methods when investigating metabolic responses to different food products to determine differences between testing methods on health outcomes.

The primary aim of this study was to investigate the metabolic responses, specifically glucose and insulin incremental area under the curve (iAUC), following consumption of 50g of digestible carbohydrate from native wheat starch (NWS) crackers (Midsol™ 50) and RS4 crackers (Fibersym® RW). The secondary aim of this study was to investigate the metabolic responses to both crackers using the FDA standard testing protocol, where both crackers were matched for digestible carbohydrate, as well as the marketplace substitution method where RS4 was substituted in place of digestible carbohydrate. The marketplace substitution method was investigated using 35g of crackers on a final product weight basis, representing an amount that is typically consumed according to the FDA (8). We hypothesized that the Fibersym® RW cracker would elicit a reduced glucose and insulin response compared to the NWS crackers at both the 50g digestible carbohydrate dose and the 35g by weight condition, thus indicating a positive metabolic response to RS4.

Materials and methods

Participants

Fourteen apparently healthy participants (ages 20–38yrs) with no history of diagnosed health conditions completed this study. Participants completed a medical history questionnaire

to determine eligibility in the study. Participants were excluded from participation if they had a baseline fasting blood glucose ≥100mg/dL, consumed a diet high in dietary fiber (>50g/day), were current smokers or smoked within the last six months, were pregnant or lactating, had an allergy to wheat or gluten, or had any diagnosed health conditions that may affect metabolism. Written and oral consent were obtained from all participants. This study was approved by the Institutional Review Board for Research Involving Human Subjects at Kansas State University IRB#8740 and conformed to the Declaration of Helsinki.

Experimental Design

This study was conducted as a single-blinded randomized-controlled crossover trial, where all participants underwent all testing conditions. Participants were randomly assigned to a series of five randomly ordered conditions, blocked in a Latin-square design. The conditions were: 1) 50g dextrose beverage (50DEX; Trutol®50 glucose-tolerance beverage), 2) 50g of digestible carbohydrate from NWS crackers (50NWS), 3) 50g of digestible carbohydrate from RS4 and NWS crackers (50RS4), 4) 35g of NWS crackers by weight (35NWS), and 5) 35g of RS4 crackers by weight (35RS4). Further details for the cracker conditions are outlined in Table 2.1. All testing sessions were performed at the Physical Activity and Nutrition Clinical Research Consortium (PANCRC) at Kansas State University, Manhattan, Kansas. All participants completed all five conditions, each following a 10–12 hour fast, with a minimum of 48 hours between sessions. To ensure consistency in responses, participants were asked to maintain their typical dietary and physical activity habits throughout the study. Additionally, participants were asked to write down what they ate the night prior to their first testing session and were reminded to consume this same meal prior to subsequent testing sessions. Inclusion criteria included: 1) apparently healthy adults with no diagnosed health conditions, 2) non-smokers, 3) not consuming

a high or low fiber diet (> 50g/day or < 5g/day), 4) lack of a wheat or gluten allergy. Participants who did not meet all inclusion criteria were excluded from participation. Participants who met the inclusion criteria at the first visit were enrolled in the study and subsequently completed pre-trial assessments of height, weight, and waist circumference, followed by an oral glucose tolerance test (OGTT) protocol. Next, participants completed a postprandial assessment for one of the five randomized conditions. The remaining four visits were performed using the postprandial assessment only. Satiety was measured via the Holt Satiety Questionnaire at baseline, 30, 60, 90, and 120 minutes during each testing session, while adding a Dual-energy X-ray Absorptiometry (DXA) scan at the final testing session to measure body composition.

Postprandial Assessment Protocol

Upon enrollment into the study, and completion of pre-trial measurements, at each session an indwelling catheter was inserted in the antecubital vein in the forearm by a researcher trained in phlebotomy. A 24-gauge safelet intravenous (IV) catheter (Exel International, Redondo Beach, CA, USA) was used to maintain an open port throughout the 2-hour testing session. A steady infusion of 0.9% NaCl was used to maintain catheter function and was fixed in place via tegaderm film (3M Healthcare, Neuss, Germany). A 3mL syringe (BD, Franklin Lakes, NJ, USA), attached to a 3-way stopcock (Fisher, Hanover Park, IL, USA), was used to clear the IV line of saline prior to whole blood collection. A 5mL syringe (BD, Franklin Lakes, NJ, USA) was then used to collect whole blood at each time point. Once the baseline blood sample was drawn, participants were instructed to consume their cracker condition or glucose tolerance beverage within a 15-minute period. The 2-hour postprandial assessment started upon completion of the final swallow for every condition to minimize the differences in consumption

time, and to add control to the start time among all participants. Approximately 500mL of water was provided at each session and was kept constant throughout the duration of the study.

Analytical Procedures

To determine blood glucose, blood samples were taken at baseline and at 15, 30, 45, 60, 90, and 120 minutes following consumption of the randomized condition. To determine insulin, samples were taken at baseline, 30, 60, and 120 minutes. Whole blood samples were drawn into a 5mL syringe (BD). A small sample of whole blood was expelled from the 5mL syringe and whole blood glucose was measured using a Bayer Contour Glucose Monitoring System. Samples were measured in duplicate, or until two readings from the glucose monitoring system were within five units of agreement (mg/dL). The final value used for analysis was the mean of two measurements that were within five units of agreement. Upon completion of the glucose measurement, the remaining whole blood was expelled into a 6mL Vacutainer test tube coated with EDTA (BD, Franklin Lakes, NJ, USA). Blood samples were centrifuged at 1800 x *g* at room temperature for 12 minutes. The plasma was then pipetted into three aliquots in 0.6mL microcentrifuge tubes (Fisher, Hanover Park, IL, USA) and stored at -80˚C until study completion (~6mo). Upon completion of data collection, one aliquot of plasma was shipped to the Radioimmunoassay and Biomarkers Core of the University of Pennsylvania Diabetes Research Center (DRC) to determine plasma insulin using double-antibody radioimmunoassay in duplicate (EMD, Billerica, MA). The insulin intra-assay coefficient of variation (CV) was 4.99% and inter-assay CV was 11.3%. The remaining two aliquots of plasma, intended for insulin analysis, were stored in case of shipment failure or in cases where analysis of samples yielded errors. Where catheter insertion was not possible, or upon failure of the IV line once inserted, blood glucose was collected via finger stick, whereas insulin was not collected. Among all

instances where catheter insertion was not possible, participants refused additional catheter insertions primarily due to needle discomfort. Blood glucose was not used for data analysis in these situations, as it was determined that these methods were statistically different. Therefore, there were missing data for participants on days where catheter failure occurred.

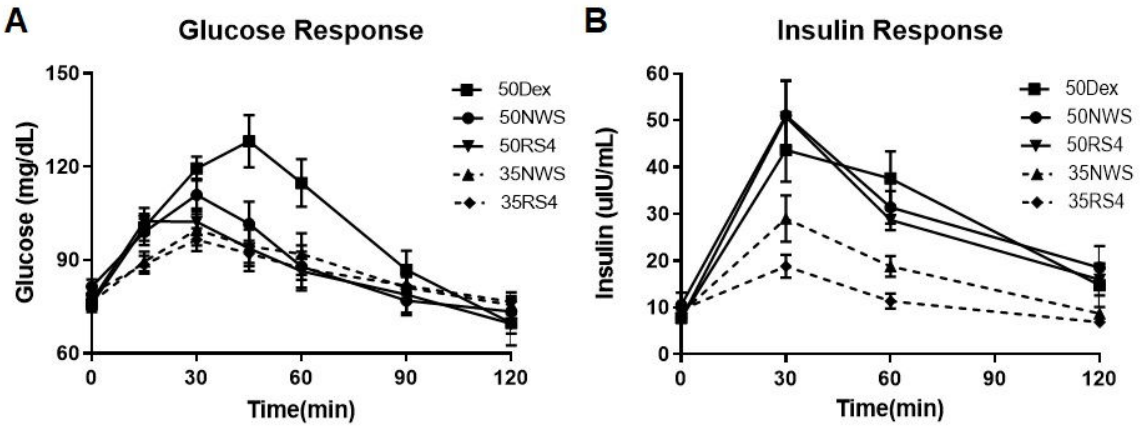
Analytic Parameters

Peak parameters (mg/dL) were defined as the highest level of glucose or insulin observed during the 2-hour testing session. Baseline-to-peak (mg/dL) was defined as the difference between the peak value and the baseline value the participant had at the start of the testing session. Time-to-peak (min) was defined as the amount of time it took for the participant to reach the peak value observed during the testing session.

The estimated means and confidence intervals from the models are reported in the text and iAUC, peak, baseline-to-peak, and time-to-peak were analyzed for glucose and insulin. A final sample size of n=13 was determined to be sufficient to detect effects based on data from Al-Tamimi et al 2010. Estimated values were converted back to the original units using the emmeans package in R and raw data are graphed (

Figure 2.1. Postprandial glycemic and insulinemic responses to dextrose and all cracker conditions during the 2hr test period. Data represent means and standard errors.

CAPTION: A) Glucose response (mg/dL) B) Insulin response (µIU/mL). FDA standard testing conditions: 50Dex = 50g dextrose; 50NWS = 68.33g NWS cracker (delivering 50g digestible carbohydrate); 50RS4 = 61.27g RS4+NWS crackers (delivering 50g digestible carbohydrate). Marketplace substitution conditions: 35NWS = 35g NWS cracker; 35RS4 = 35g RS4 cracker.



) to assist with interpretability.

Body Composition Measures and Questionnaires

Height, weight, and waist circumference measures were collected in duplicate and the mean values were used for data analysis. Height was measured to the nearest 0.1cm using a portable stadiometer (Invicta Plastics, Leicaster, England). Participants were measured with heels, buttocks, and shoulders touching the flat upright surface. Weight was measured to the nearest 0.1kg with an electronic scale (Pelstar LLC, Alsip, IL, USA). Waist circumference was measured to the nearest 0.1cm using a tape measure by locating the top of the iliac crest. Measurements were taken at the end of the participant's normal exhalation. Dual-energy X-ray Absorptiometry (DXA) was used to determine body composition (GE prodigy, Lunar-General Electric, Madison, WI). Participants were asked to take jewelry, shoes, and anything with metal off before lying in a supine position for approximately 6–10 minutes for the DXA scan. Satiety was measured throughout each trial using the Holt Satiety Scale. This 7-point visual analog scale has equally spaced options that include extremely hungry, hungry, semi-hungry, no particular feeling, semi-satisfied, satisfied, and extremely satisfied. The Holt Satiety Scale was previously used to determine the Satiety Index of common foods and used in this study to measure satiety to the cracker conditions (40). The International Physical Activity Questionnaire-Short Form (IPAQ-SF) was used to assess physical activity levels for each participant. This questionnaire covers four domains of physical activity, including work related, transportation, household/gardening, and leisure time physical activity. The questions include frequency factors, number of days per week, and time conducting various activities including sitting, walking, and moderate and vigorous intensity physical activity. The primary use of the IPAQ-SF in this study was to determine whether physical activity levels changed during the study.

Dextrose Beverage and Cracker Details

The glucose tolerance test beverage provided 50g of dextrose (Thermo Fischer Scientific; Catalog Number: 401074P). The crackers were produced locally at the American Institute of Baking (Manhattan, KS). The four cracker conditions were made using the same ingredients other than the type of starch. The RS4 cracker was made by replacing native wheat starch in a cracker with a type 4 resistant wheat starch while the NWS cracker was made solely from native wheat starch, all the while maintaining protein content constant by adding wheat gluten. The 50NWS cracker condition was tested using 68.33g NWS providing 50g of digestible carbohydrate from NWS. The 50RS4 cracker condition was tested using a combination of 35g RS4 + 61.27g NWS providing 50g of digestible carbohydrate. The 35NWS cracker was tested as 35g of NWS cracker by final product weight, and 35RS4 was tested using 35g of RS4 cracker by final product weight. All ingredients were food grade and generally recognized as safe (GRAS). Both the native wheat starch (Midsol^{TM} 50) and resistant starch (Fibersym$^{®}$ RW) were provided by MGP Ingredients Inc. (Atchison, KS), with food grade documentation (Table 2.1). Finished NWS and RS4 crackers were nearly identical and could only be distinguished by the label on their designated package.

Statistical Analysis

Data analyses were conducted in R, version 3.5.3 (41). Repeated measures mixed-effects regression models were conducted for each of the outcome variables to account for missing data. Primary outcomes were glucose iAUC and insulin iAUC. Secondary outcomes were glucose and insulin peak, baseline-to-peak, and time-to-peak. Main effects of Condition, Dose, and Condition×Dose were included as fixed effects in all primary and secondary analyses. Condition (NWS, RS4) and Dose (50g digestible carbohydrate, 35g by weight) were effect coded. Subject

was included as a random effect to account for individual differences. Two groups of analyses were used to determine effects of Condition and Dose. Experimental conditions were analyzed in groups based on the method of preparation. The first analysis included the FDA standard testing conditions (50Dex, 50NWS, 50RS4). The second analysis included the marketplace substitution method conditions (35NWS, 35RS4) and the 50g cracker conditions (50NWS, 50RS4). To determine the effect of Condition, 50Dex, 50NWS, and 50RS4 were analyzed together as these conditions were matched for digestible carbohydrate. To determine effects of Dose, 50NWS, 50RS4, 35NWS, and 35RS4 were analyzed together and 50Dex was excluded from this analysis because there was not a low dose of dextrose used as a condition in this study. Posthoc comparisons were conducted for significant effects using the emmeans package in R. Outcome variables were transformed for normality according to boxcox analyses using the MASS package in R. Specifically, a boxcox analysis was conducted which informed the type of transformation that should be used to transform each variable. Following the transformation of data, a histogram and residual plot were generated to verify the normal distribution of data. If more than one transformation was identified using the boxcox analysis, the transformation used was aligned according to similar variables. For example, if glucose iAUC was transformed via a square root transformation and the boxcox for insulin iAUC showed a log and square root transformation as potential options, both transformations were performed and histograms and residual plots were generated to observe the normal distribution of data. If one of the transformations looked drastically in favor of another potential transformations, then that transformation was used. However, if both transformations yielded a similar distribution of data, the square root transformation was used to align the insulin iAUC data with the glucose iAUC transformation. Glucose and Insulin incremental area under the curve (iAUC) were obtained using GraphPad

Prism version 8.1.0 with the trapezoidal method (GraphPad Software, Inc. La Jolla, CA, USA). Statistical significance was set at $p<0.05$. Glucose Peak, insulin peak, and insulin baseline-to-peak were log-transformed. Glucose iAUC, insulin iAUC, glucose baseline-to-peak, and insulin time-to-peak were square-root transformed.

Missing values for glucose and insulin were due to IV catheter failure or processing issues with insulin analysis of samples on seven occasions out of the 70 visits from all participants and were distributed across all cracker conditions. In all instances where catheter failure occurred, participants had the option to have a new catheter inserted for data collection, or to refuse reinsertion of the catheter and continue the visit with capillary finger prick blood collection. All data collected via the catheter, during visits with catheter failure, were included in data analysis if they had passed a presumed peak following the 1-hour timepoint.

Results

Fourteen apparently healthy adults (9 male; 5 female) between the ages of 20–38yrs completed the study, with one participant withdrawing following the second testing session (Figure 2.2). Demographic, anthropometric, baseline glucose, and baseline insulin results are shown in Table 2.2. Seven subjects were classified as overweight or obese (BMI ≥ 24.9).

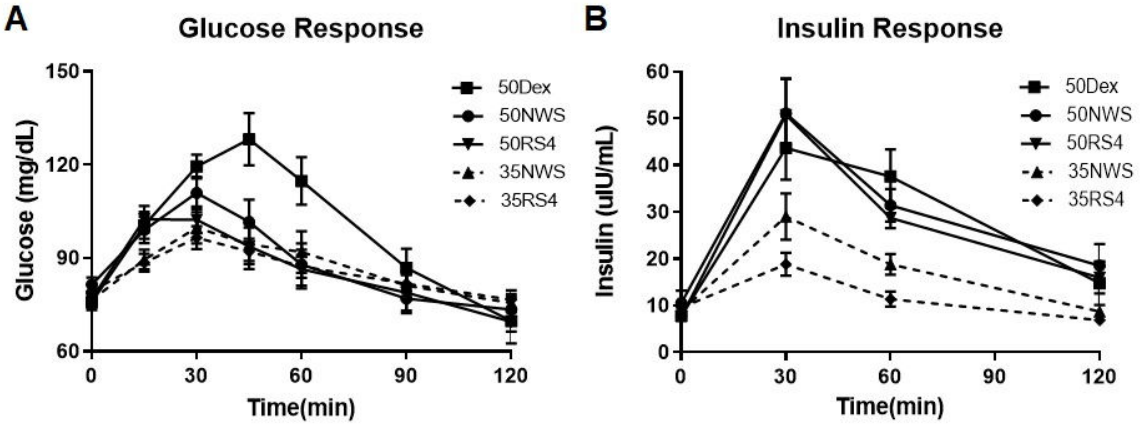
Comparison of 50g FDA standard testing method conditions

When comparing the FDA standard testing protocol 50g conditions (50Dex, 50NWS, 50RS4) matched for digestible carbohydrate, consumption of both the 50NWS and 50RS4 crackers resulted in a lower glucose iAUC, glucose peak, and glucose baseline-to-peak when compared to 50Dex (*ps*<0.001) (

Figure 2.1. Postprandial glycemic and insulinemic responses to dextrose and all cracker conditions during the 2hr test period. Data represent means and standard errors.

CAPTION: A) Glucose response (mg/dL) B) Insulin response (μIU/mL). FDA standard testing conditions: 50Dex = 50g dextrose; 50NWS = 68.33g NWS cracker (delivering 50g digestible

carbohydrate); 50RS4 = 61.27g RS4+NWS crackers (delivering 50g digestible carbohydrate). Marketplace substitution conditions: 35NWS = 35g NWS cracker; 35RS4 = 35g RS4 cracker.



and Table 2.3). Glucose iAUC was approximately 58% lower, glucose peak was approximately 15% lower, and glucose baseline-to-peak was approximately 45% lower for both cracker conditions when compared to 50Dex. However, no statistically significant differences were observed between the 50NWS and 50RS4 crackers (*ps*>0.05). No differences were observed for glucose time-to-peak between 50g conditions (*ps*>0.05). No differences were observed between 50g conditions for insulin iAUC, insulin peak, insulin baseline-to-peak, or insulin time-to-peak (*ps*>0.05). Results for 50g comparisons are displayed in Table 2.3.

Comparison of 35g marketplace substitution method

Marketplace substitution cracker comparisons (35NWS, 35RS4) did not include the comparison with 50Dex. When comparing the 35g conditions, there was no effect of dose (p=0.32), condition (p=0.17), or dose*condition(p=0.22) for glucose iAUC, glucose peak, glucose baseline-to-peak, or glucose time-to-peak (*ps*>0.05). However, there was a significant effect of dose (p<0.0001), condition (p<0.0001), and dose*condition (p<0.01) for insulin iAUC. The 35RS4 condition elicited a lower insulin iAUC, insulin peak, and insulin baseline-to-peak compared to the 35NWS condition (*ps*<0.01). Insulin iAUC was 87% lower for the 35RS4

condition compared to the 35NWS condition. Insulin peak was 39% lower for 35RS4 compared to 35NWS and insulin baseline-to-peak was 66% lower for 35RS4 compared to 35NWS. No differences were observed for insulin time-to-peak (p>0.05). Results for the two testing protocols (FDA and marketplace substitution) are compared in Table 2.3.

Discussion

In light of the prevalence of metabolic disease globally, and the promising research on resistant starches thus far, the primary aim of this study was to investigate glycemic and insulinemic responses to Fibersym® RW (RS4) crackers compared with native wheat starch (NWS) crackers. As part of this aim, we compared RS4 and NWS under two different testing protocols, the FDA standard testing protocol and the marketplace substitution method. When comparing the 50g conditions using the FDA standard testing protocol, glycemic responses to RS4 and NWS crackers were similar over a two-hour testing period but differed considerably from dextrose. Similarly, the insulin responses did not differ between 50g cracker conditions. We hypothesized that the RS4 cracker would elicit a lower glycemic and insulinemic response compared to the NWS cracker, regardless of the testing protocol used. The null results can be explained given that the RS4 in the 50RS4 cracker condition was substituted for digestible carbohydrate in the 50NWS cracker condition, in accordance with the FDA standard testing method. This addition led to a 50RS4 condition containing a similar amount of digestible carbohydrate compared to the 50NWS condition, which could be expected to elicit a similar glycemic and insulinemic response. We hypothesize that these results may be due to the baking process used for the crackers, considering previous testing on RS4 in a nutrition bar, in accordance with the FDA standard testing protocol, has shown reduced glucose and insulin responses following preparation of nutrition bars as described previously (15).

When examining crackers made using the marketplace substitution method, without matching for digestible carbohydrate (35NWS, 35RS4), the insulinemic response was 87% lower for the 35RS4 cracker compared to the 35NWS cracker. Although the glycemic response to 35RS4 was ~45% lower compared to 35NWS, there was not a statistically significant difference between 35g cracker conditions. This was primarily due to large within-individual variability within both 35g cracker conditions (Table 2.3). The lack of statistical differences in glycemic response between the 35g cracker conditions was surprising and did not support our original hypothesis. Previous research has reported a reduced glycemic response to RS4 following both the FDA standard testing method and the marketplace substitution method (15, 22-24). Reduced insulin responses have been previously reported when investigating foods containing higher amounts of fiber (31, 42-44). Additionally, resistant starch has been shown to elicit a reduced insulinemic response compared to foods matched for digestible carbohydrate or by weight (15, 45-47). Our results agree with these studies as we showed reduced insulin responses following acute consumption of an RS4 cracker. Of particular interest, reduced insulin responses for RS4 and other types of RS, even when compared with foods eliciting similar glycemic responses, is a recent finding that indicates a reduced load on the glucose-insulin system showing benefit for the consumption of foods containing RS (16, 48). Additional research is needed to elucidate a potential beneficial effect of RS on insulin sensitivity.

The type of testing method for food products is important to consider, especially when these products are being tested to obtain FDA food label classifications. The current standard testing methods for products seeking a fiber food label classification are to match both test and control foods by adding the test ingredient on top of the control food, or by substituting the ingredient for a similar ingredient, all the while matching for digestible carbohydrate. From a

physiological perspective, the addition of fiber on top of the 50g carbohydrate amount may not reduce a metabolic response to the test food; it might simply delay that response or have no effect, depending on the mechanism of action. Furthermore, when investigating high fiber foods, the amount of product needed to elicit a marked change in glycemic or insulinemic response may not be known. This is particularly true when resistant starches are part of the product formulation, as researchers typically test at the 50g digestible carbohydrate amount to preserve the power to detect differences where differences might be small. However, the 50g carbohydrate amount represents a challenge for glucose and insulin testing in high fiber foods because 50g of carbohydrate is likely to elicit a large glucose and insulin response, irrespective of fiber content.

The FDA testing methods may not represent how an individual is likely to consume a fiber ingredient in a fortified food. The FDA standard testing method of matching for digestible carbohydrate raises an additional concern for testing high fiber foods due to the low digestible carbohydrate amount some products contain. Low amounts of digestible carbohydrate in products lead to testing that may use drastically larger quantities of food than would typically be consumed by individuals. Further, this testing method could lead to multiple treatments that contain drastically different amounts of food. Theoretically, this could yield results that are primarily impacted by different mechanisms, and results that are potentially irrelevant to consumers if the products are not consumed in large quantities. It is important that the consumer knows how a given product may elicit a beneficial or harmful metabolic response. Consumers may be trying to lose weight, improve metabolic health, or substitute “unhealthful” products in place of less healthy options they would normally choose. Accurate information regarding more

true-to-life metabolic responses to food products should be provided to consumers so that they can make informed choices regarding which products they would like to consume.

One argument in favor of using the FDA standard testing method is that it uses an approach that matches digestible carbohydrate, such that multiple treatments can be compared while adding a layer of control over the digestible carbohydrate amounts of treatments. If beneficial metabolic outcomes are observed following acute consumption of high-fiber products, then theoretically they would carry over regardless of how consumers eat the products. However, beneficial metabolic effects may not be seen, compared against competing products, when these products are tested using lower amounts of the matched digestible carbohydrate. There may be a minimum dose of fiber necessary to elicit beneficial metabolic outcomes. According to the FDA Reference Amounts Customarily Consumer Per Eating Occasion, a typical serving size for crackers is approximately 30 grams per eating occasion (21). This reported amount is based on a per weight basis, meaning the product would be made using the marketplace substitution method and would represent a product on the shelf of a grocery/convenience store. These differences observed between standard testing methods and the more true-to-life marketplace substitution testing method highlight an important concern regarding how foods are tested compared to how they are sold and consumed.

There are several strengths of the current study that should be considered when evaluating the results. Strengths of this study include the novelty of the study design to parse apart differences between the FDA standard testing method and the more true-to-life marketplace substitution testing method where fiber is substituted in place of other carbohydrate ingredients, but not matched for digestible carbohydrates. This study design elucidates a need for further evaluation of the current FDA standard testing methods for glucose and insulin responses

required for fiber food-label classification. The single-blinded randomized-controlled crossover trial design is another strength of this study, as it allowed for multiple comparisons of cracker types and the method used to produce the cracker conditions, while minimizing within subject heterogeneity. The primary limitations of this study include the absence of a 35g dextrose comparison, inhibiting our ability to directly compare the FDA standard testing method to the marketplace substitution method for this carbohydrate amount. This condition was not included in order to limit participant burden. The small sample size of human subjects is also a potential limitation. The use of the Bayer Contour glucometer is another potential limitation as this is a consumer geared device that may not report the most accurate absolute glucose values. Although this is a potential limitation, this was accounted for with multiple measurements to ensure a more accurate measure of absolute glucose values. A final limitation is the use of multiple comparisons, which inherently increases the potential for type I error. Future studies should investigate differences between FDA standard testing methods and marketplace substitution methods with respect to glycemic and insulinemic responses using different products and different carbohydrate doses. Additionally, broader populations should be investigated to determine whether similar effects are observed, including populations with chronic conditions such as diabetes.

Our results indicate differential outcomes using an FDA approved protocol for testing of the glycemic and insulinemic responses to an RS4 and NWS cracker as opposed to a marketplace substitution protocol. The FDA standard testing method reported no differences between crackers at the 50g digestible carbohydrate amount. However, there was a significantly lower postprandial insulinemic response for the RS4 cracker using the marketplace substitution method, matched by weight (35g) and not by carbohydrate amount, even with similar

postprandial glycemic responses as compared with the NWS cracker. Given the potential of fiber, and more specifically resistant starch, for improving metabolic responses, current testing for FDA claims on food labels should be evaluated further to determine their appropriateness as compared with using more true-to-life testing amounts.

Table 2.1. Nutrient composition of each condition (per 100 grams)

	50NWS	50RS4	35NWS	35RS4
Ash (g)	1.0	1.7	0.5	0.7
Moisture (g)	3.4	4.2	1.7	1.2
Carbohydrate (g)	50.3	71.3	25.8	26.1
Digestible carbohydrate (g)	50.0	50.0	25.6	5.3
Dietary fiber (g)	0.3	21.3	0.2	21.0
Fat (g)	7.6	10.8	3.9	4.0
Protein (g)	6.0	8.5	3.1	3.1
Total calories (kcal)	292.0	331.4	149.6	69.5
Final product weights provided to participants (g)	68.3	35.0 RS4 + 61.3 NWS (96.3 total)	35.0	35.0
Digestible carbohydrate was calculated as Carbohydrate (g) minus Dietary fiber (g).				

Table 2.2. Baseline participant characteristics of individuals who completed the study. Results are reported as Mean ± SD.

	All (n=14)
Sex (Male;Female)	9;5
Age (yr)	24.6 ± 4.7
Height (cm)	175.5 ± 8.8
Weight (kg)	76.6 ± 16.8
Waist Circumference (cm)	87.2 ± 11.0
BMI (kg/m^2)	24.8 ± 10.1
Body Fat (DXA, %)	19.3 ± 10.1
Fasting Glucose (mg/dL)	79.3 ± 9.1
Fasting Insulin (μIU/mL)	1.1 ± 0.6
DXA: Dual-energy X-ray Absorptiometry scan for body fat percentage.	

Table 2.3. Summary of means by testing method. p-values indicate comparisons between cracker conditions.

Method		Dextrose Control	FDA Standard Testing Method			Substitution Method (True-to-life)		
			50g matched digestible carbohydrate			35g matched by weight		
	Variable	50Dex Mean (95%CI)	NWS Mean (95%CI)	RS4 Mean (95%CI)	p^*	NWS Mean (95%CI)	RS4 Mean (CI)	p^*
Glucose	iAUC (mg/dL x 2hr)	3016 (2115, 4076)	1251 (720, 1929)	1276 (739, 1960)	.99	1317 (677, 2169)	701 (262, 1351)	.37
	Peak (mg/dL)	134 (122, 147)	115.4 (106.6, 124)	111.1 (102.3, 120)	.61	104.3 (94.1, 114)	97.5 (87.4, 108)	.70
	Baseline-to-peak (mg/dL)	56.1 (42.5, 71.5)	30.2 (21.7, 40.2)	32.0 (23.2, 42.2)	.98	28.2 (18.5, 39.9)	16.9 (9.61, 26.2)	.20
	Time-to-peak (Minutes)	34.3 (27.0, 41.6)	28.9 (21.9, 36.0)	27.9 (20.8, 34.9)	.99	46.1 (36.4, 55.8)	36.4 (26.7, 46.1)	.50
Insulin	iAUC (µIU/mL x 2hr)	2470 (1836, 3199)	2311 (1820, 2861)	2330 (1836, 2882)	.99	697 (397, 1080)	92 (10, 259)	<.01
	Peak (µIU/mL)	42.3 (32.2, 55.6)	47.9 (36.5, 63.0)	45.8 (34.9, 60.2)	.58	21.7 (16.1, 29.4)	13.3 (9.8, 17.9)	<.01
	Baseline-to-peak (µIU/mL)	28.3 (17.1, 42.4)	38.0 (26.8, 53.9)	36.9 (26.0, 52.3)	.99	13.9 (9.34, 20.8)	4.75 (3.18, 7.09)	<.01

	Time-to-peak (Minutes)	49.3 (39.8, 58.8)	36.4 (29.4, 43.4)	34.3 (27.3, 41.3)	.97	39.3 (29.8, 48.8)	40.0 (30.5, 49.5)	.99

Testing method comparisons with the Dextrose control beverage are reported in the results section. Statistical significance was set at $p<0.05$.

Figure 2.1. Postprandial glycemic and insulinemic responses to dextrose and all cracker conditions during the 2hr test period. Data represent means and standard errors.

CAPTION: A) Glucose response (mg/dL) B) Insulin response (µIU/mL). FDA standard testing conditions: 50Dex = 50g dextrose; 50NWS = 68.33g NWS cracker (delivering 50g digestible carbohydrate); 50RS4 = 61.27g RS4+NWS crackers (delivering 50g digestible carbohydrate). Marketplace substitution conditions: 35NWS = 35g NWS cracker; 35RS4 = 35g RS4 cracker.

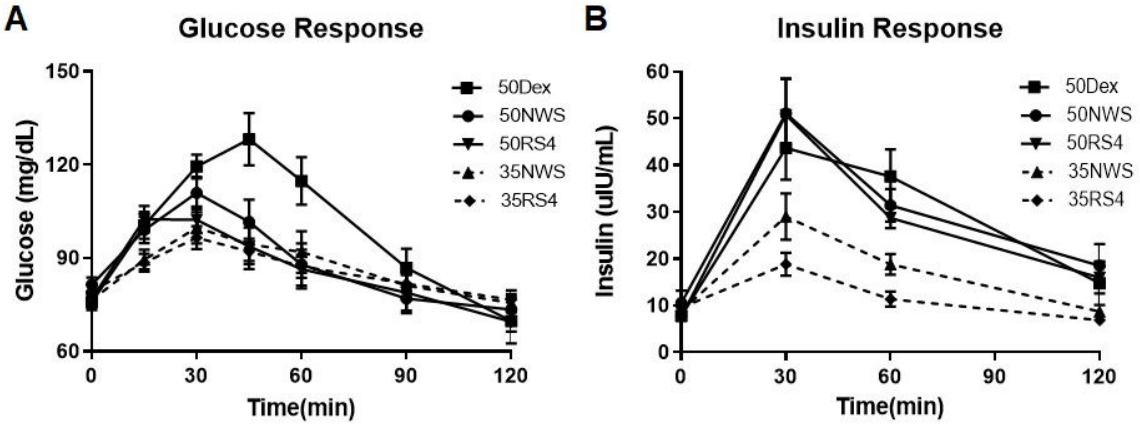
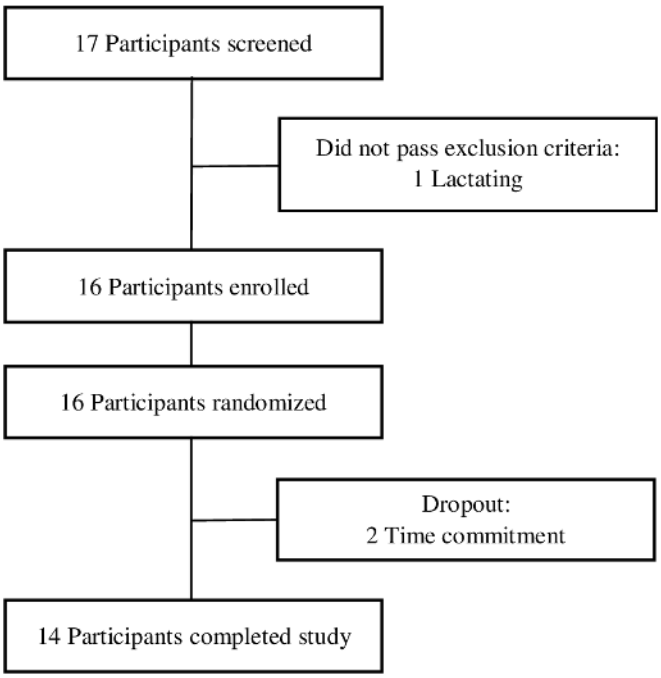


Figure 2.2. Consort Diagram



Chapter 3 - Glycemic and insulinemic responses of healthy humans to a nutrition bar with or without added Fibersym® RW, a cross-linked RS4-type resistant wheat starch

High dietary fiber consumption has been associated with reduced risk of developing non-communicable chronic disease, including cardiovascular disease and diabetes, among others (31, 49). Further, recent research suggests that dietary fiber may play a role in treating obesity-related disorders (50). Despite numerous studies showing benefits of dietary fiber, national levels of consumption remain drastically low with 90% of American women and 97% of American men not meeting recommended fiber intake (7). A recent review suggests that consuming enough dietary fiber, at "advisable" amounts between 30-40g, has several consistent benefits for human health and that including fiber in manufactured carbohydrate containing products has the potential to enhance public health and aid in the prevention of non-communicable chronic disease (51).

One method of adding fiber to food products is through the addition of resistant starch type 4 (RS4), a human created fiber primarily used as a substitute for flour in food products, to add fiber and reduce the digestible carbohydrate content of marketplace food products. Existing randomized controlled trials have shown beneficial effects of RS4 intake on glycemic and insulinemic responses (15-25). To test the benefits of RS4 for fiber food label classification, researchers often test at a 50g amount of digestible carbohydrate (15, 20, 27). In a research setting, this amount of digestible carbohydrate typically requires a larger bolus of food than is typically consumed by consumers in a single sitting. For this reason, there may be differential responses observed at these testing amounts that may not translate to a typical, standard serving

size for consumers (20). There is limited research, using a Food and Drug Administration (FDA) approved testing approach for fiber food label classification, incorporating lower doses (<50g) of digestible carbohydrate that are likely to represent more typical consumption amounts. However, a recent randomized controlled trial reported improvements in glycemic and insulinemic responses at a lower amount of digestible carbohydrate (35g), a dose more typical of a consumer portion, regardless of the dose of RS4 (17).

In order to elucidate the effects of RS4 on postprandial glycemic and insulinemic responses, we conducted an investigation of RS4 provided in the form of a nutrition bar, used in similar studies (15, 17), at a high dose (50g) and a low dose (30g) of digestible carbohydrate. The primary purpose of this randomized, single-blinded, crossover study was to determine whether glycemic and insulinemic responses were different when using an amount of digestible carbohydrate more commonly used in research as compared with a consumer-friendly portion of digestible carbohydrate more typical of a marketplace food product. In addition, we sought to investigate the glycemic and insulinemic responses to RS4 as compared to a standard puffed wheat bar (PWB) and a dextrose control at both the low and high doses (50g and 30g) of digestible carbohydrate. We hypothesized that the Fibersym® RW bar would elicit lower glucose and insulin responses compared to the PWB and dextrose control at both matched doses.

Materials and methods

Participants

Fifteen apparently healthy participants (ages 20–38yrs) with no history of diagnosed health conditions completed this study (Participant characteristics are displayed in

Table 3.2). A power analysis was conducted using data from Al-Tamimi, Seib (15), indicating that a total sample of 12 participants would be needed to detect large effects (d = 1.7) at 80% power and alpha at 0.05 when comparing 50g digestible carbohydrate PWB and RS4 nutrition bars. In order to account for potential participant dropout, we recruited 17 participants. All participants completed a medical history questionnaire and a screening visit to determine eligibility to participate in this study. Participants were excluded if they had a baseline fasting blood glucose ≥100mg/dL, consumed a diet high in dietary fiber (>50g/day), were current smokers or had smoked within the past six months, were pregnant or lactating, had a wheat or gluten allergy, or had any diagnosed health conditions that might have affected glycemia or insulinemia. This study was approved by the Institutional Review Board for Research Involving Human Subjects at Kansas State University (IRB #9368) and conformed to the Declaration of Helsinki.

Experimental Design

The current study was conducted as a single-blinded randomized-controlled crossover trial, where all participants experienced all treatments. Methods and procedures were modified from Steele, Maningat (20). Specifically, the timepoints of whole blood collection and the method of glucose analysis were modified.

All testing sessions were performed in the Physical Activity and Nutrition Clinical Research Consortium (PANCRC) at Kansas State University, Manhattan, Kansas. Prior to randomization, participants were required to pass an initial 2-hour screening visit following consumption of a 75g glucose tolerance dextrose beverage. In order to take part in the study, participants had to have a glucose value <100 mg/dL at baseline and <140 mg/dL at two hours following consumption of the dextrose beverage. Upon completion of screening, participants

were randomly assigned to a series of six treatments via a blocked Latin-square design. Participants completed a postprandial assessment following a glucose tolerance test protocol at each visit. The postprandial assessment was conducted as previously described in Steele, Maningat (20). Each participant consumed one randomized treatment during a given testing session. The six treatments were completed following a 10–12 hour fast with at least 48 hours between treatments and included the following: 1) 50g dextrose beverage (50DEX; Trutol®50 glucose-tolerance beverage), 2) 30g dextrose beverage (30DEX), 3) 50g of digestible carbohydrate from a PWB bar (50PWB), 4) 30g of digestible carbohydrate from a PWB bar (30PWB), 5) 50g of digestible carbohydrate from an RS4 bar (50RS4), and 6) 30g of digestible carbohydrate from an RS4 bar (30RS4). Additional details for the nutrition bar treatments are shown in Table 3.1. To reduce variability in daily postprandial responses, participants were asked to maintain dietary and physical activity habits throughout the study period. Additionally, participants were asked to record what they ate the night prior to their first treatment and were reminded to consume the same snack/meal prior to subsequent testing sessions. Satiety was measured via the Holt Satiety Questionnaire at baseline, 30, 60, 90, and 120 minutes during each testing sessions. A Dual-energy X-ray Absorptiometry (DXA) scan was conducted at the final testing session to determine body composition.

Analytical Procedures

To determine whole blood glucose and plasma insulin, whole blood was collected at baseline, 10, 20, 30, 60, 90, and 120 minutes following consumption of the randomized treatment. Whole blood samples were drawn into a 5mL syringe (BD), and a small amount was used to measure whole blood glucose using a Cholestech Cassette (Alere Cholestech LDX TC•GLU cassettes, product code 10-988). Samples were measured in duplicate, and the mean of

two measurements was used for statistical analysis. Blood processing following whole blood glucose analysis was conducted as previously described (20).

Anthropometric Measures and Questionnaires

Height and weight were measured in duplicate at the screening visit, and the mean values were used for data analysis. Height was measured to the nearest 0.1cm using a portable stadiometer (Invicta Plastics, Leicaster, England). During the measurement, participants maintained an upright posture with heels, buttocks, and shoulders touching the upright surface of the stadiometer. Weight was measured to the nearest 0.1kg with an electronic scale (Pelstar LLC, Alsip, IL, USA). Dual-energy X-ray Absorptiometry (DXA) was used to determine body composition at the final visit (GE prodigy, Lunar-General Electric, Madison, WI). Participants removed all jewelry, shoes, and other metal items prior to lying in a supine position for approximately 6–12 minutes for the DXA scan.

Satiety was measured during each treatment using the Holt Satiety Scale (40). This 7-point visual analog scale has equally spaced choice anchors that include extremely hungry, hungry, semi-hungry, no particular feeling, semi-satisfied, satisfied, and extremely satisfied. The International Physical Activity Questionnaire-Short Form (IPAQ-SF) was used to assess physical activity levels for each participant (52). This questionnaire covers four domains of physical activity including work related, transportation, household/gardening, and leisure time physical activity. The questions include frequency factors, number of days per week, and time conducting various activities including sitting, walking, and moderate and vigorous intensity physical activity. The primary use of the IPAQ-SF in this study was to verify the maintenance of physical activity levels throughout the course of the study.

Dextrose Beverage and Nutrition Bar Details

The glucose tolerance test beverage provided either 50g or 30g of dextrose (Thermo Fischer Scientific; Catalog Number: 401074P). The PWB and RS4 bars were provided by MGP Ingredients Inc. (Atchison, KS) and were formulated using the same general ingredients used in a previous study (15). Thus, all ingredients in the PWB and RS4 bars were identical except for the source of starch used. A breakdown of the nutrient composition of the nutrition bars is displayed in Table 3.1. The 50PWB treatment was tested using a 91.7g puffed wheat bar providing 50g of digestible carbohydrate from puffed wheat yielding 269.2kcals. The 50RS4 treatment was tested using a 106.4g RS4 bar providing 50g of digestible carbohydrate from RS4 yielding 243.0kcals. The 30PWB treatment was tested using a 55.0g puffed wheat bar yielding 161.5kcals and the 30RS4 treatment was tested using 63.8g of RS4 bar yielding 145.7kcals. All ingredients were food grade and generally recognized as safe. The PWB and RS4 bars were distinguishable from one another as the puffed wheat had a puffed appearance compared to the non-puffed nature of RS4. Participants were not aware of the specifics of the treatment being consumed (i.e., which contained RS4, and which did not) until all nutrition bar treatments had been completed.

Statistical Analysis

Data analyses were performed using R, version 3.5.3 (41). A single repeated measures mixed-effects linear regression model was used to determine differences between dose amounts (Dose), treatment type (Treatment), and the interaction between dose and all treatments. Treatment, Dose, and Treatment×Dose were included as fixed effects. Treatment (DEX, PWB, RS4) and Dose (50g digestible carbohydrate, 30g digestible carbohydrate) were effect coded. Participant was included as a random effect to account for individual differences. Statistical significance was set at $p<0.05$. Posthoc comparisons were performed for significant effects with

the emmeans package in R. Outcome variables were transformed for normality according to boxcox analysis using the MASS package in R. Following the transformation of data, histograms and residual plots were used to verify the normal distribution of data. If more than one transformation was identified using the boxcox analysis, the transformation used was aligned across similar variables. The analytic parameters and calculations for peak values and HOMA-IR are described in Steele, Maningat (20). Glucose and insulin incremental area under the curve (iAUC) were calculated in R using the trapezoid method. Glucose iAUC and insulin iAUC were square-root transformed. Glucose peak, insulin peak, glucose time-to-peak, insulin time-to-peak, glucose baseline-to-peak, insulin baseline-to-peak, insulin:glucose ratio, and HOMA-IR were log-transformed. Estimated values were converted back to the original units using the emmeans package in R and are reported in the text and tables. Raw data are graphed.

Results

Fifteen apparently healthy adults (8 male; 7 female) between the ages of 20–38yrs, with no diagnosed health conditions, completed this study. A total of two participants withdrew from the study following one and two sessions (Figure 3.1). Demographic, anthropometric, baseline glucose, and baseline insulin values are shown in Table 3.2. Seven subjects were classified as overweight or obese (BMI $\geq$ 25.0kg/m^2) and the remainder were classified as normal weight (<25.0kg/m^2).

Means and 95% confidence intervals for dose and treatment comparisons are reported in Table 3.3. Postprandial glucose and insulin data for each treatment are displayed in Figure 3.2. There was a significant main effect of dose and treatment for glucose iAUC, insulin iAUC, glucose peak, insulin peak, glucose baseline-to-peak, and insulin baseline-to-peak. Specifically, 30g responses were significantly lower than 50g responses. In addition, RS4 treatment responses

were significantly lower compared with PWB and DEX responses at both the 30g and 50g doses. There was no significant interaction for any of the outcome measures; the means and CIs of each treatment for each dose are reported in Table 3.4.

For HOMA-IR, there was a significant main effect of dose, $p<0.001$, such that the 50g dose resulted in lower HOMA-IR values compared with 30g doses. There was no effect of treatment, dose, or an interaction for insulin to glucose ratio (ps<0.05).

Discussion

Given the prevalence of non-communicable chronic diseases that may be partially mitigated by increasing fiber consumption and the promising preliminary research on resistant starch type 4 (15, 17-25), the primary aim of this study was to investigate the effects of two doses of available carbohydrate containing Fibersym® RW (RS4) on glycemic and insulinemic responses when compared with puffed wheat bar (PWB) and dextrose. The current study utilized the FDA approved testing method of matching digestible carbohydrates to determine whether glycemic and insulinemic responses were similar using a 50g dose of digestible carbohydrate in addition to a 30g dose of digestible carbohydrate, representing an amount similar to a typical serving size a consumer may experience daily (8). Significantly lower glucose and insulin iAUC were observed at both doses of RS4 when compared to a puffed wheat bar and a dextrose beverage. These results suggest that potential beneficial metabolic responses for nutrition bars containing RS4 are consistent in larger as well as lower doses of digestible carbohydrate that are more in line with the carbohydrate content for serving sizes of nutrition bar products purchased by consumers.

Secondary outcomes followed a similar pattern, indicating lower values following RS4 consumption for glucose and insulin peak as well as glucose and insulin baseline-to-peak when

compared with PWB and Dextrose. These reduced postprandial values, considered alongside the reductions in glucose and insulin iAUC, indicate consistent glycemic and insulinemic reductions following consumption of RS4 nutrition bars as compared with PWB and dextrose control beverages matched for digestible carbohydrate.

Research investigating multiple RS4 doses within a single study is scarce. Of the research that is available, inconsistent results are reported for glycemic and insulinemic responses when matching for digestible carbohydrate (17, 27). The existing research suggests that there are no differences in glycemic or insulinemic responses across different doses of RS4 when matching for digestible carbohydrate (17, 27). The current study further demonstrates that there are beneficial effects of RS4 consumption on postprandial glycemic and insulinemic responses regardless of the amount of digestible carbohydrate consumed in amounts up to 50g of digestible carbohydrate.

However, the results of these studies differed regarding the overall effects of RS4 on glycemic and insulinemic responses. One study reported no differences between RS4 and the control condition (27), consistent with Steele, Maningat (20). Du, Wu (27) used the addition method, where the RS4 dose was added on top of the formulated control product and did not report lower glycemic or insulinemic responses for an RS4 cereal bar. Another study found that RS4 elicited lower glycemic and insulinemic responses compared to the control condition (17), consistent with Al-Tamimi, Seib (15). However, Gourineni, Stewart (17) used the substitution method, where the RS4 replaced (was substituted for) a similar ingredient in the control product and the tested nutrition bars were matched for digestible carbohydrate, similar to the current study. The studies that found no differences between RS4 and the control conditions used the

add-on FDA approved method while the studies that found differences between RS4 and the control conditions used the FDA approved substitution method.

Results from the current study, along with the available results from previous investigations of differential responses following multiple RS4 doses, elucidates two potentially important factors to note regarding the impact of RS4 on glycemic and insulinemic responses. The addition of RS4 on top of a given carbohydrate containing product does not appear to negate the glycemic and insulinemic responses to other existing carbohydrate ingredients in the formulated nutrition bar. However, when the non-fiber ingredient is substituted with RS4, reductions in glycemic and insulinemic responses appear. Thus, the method of inclusion of RS4 in a given product, addition to a product vs substituted in a product, may be important for attenuations in glycemic and insulinemic responses to exist. Therefore, the formulation of the product may be important when considering the potential benefits of an RS4 product as compared to a standard non-RS4 carbohydrate containing product.

The current study has several strengths, including the use of a randomized single-blind crossover design. This study design allowed for results to be parsed apart for both doses of digestible CHO among all three treatment types, all while eliminating between subject heterogeneity. An additional strength of this study is the use of a single method of product formulation for all nutrition bar treatments. For the current study, we used the substitution method, matching for digestible carbohydrate across dose and treatment type. This method eliminates many potential limitations of the interpretation of results when comparing doses, treatment types, and dose x treatment interactions due to the absence of confounding interactions occurring between multiple carbohydrate sources observed using the add-on method. One limitation of the current study is the use of multiple comparisons, which inherently increases the

potential occurrence of a type 1 error. A second limitation was the finding of a significant effect of dose for HOMA-IR, a measure based on fasting values that should not be influenced by the experimental conditions, indicating a type 1 error. We designed the study to account for daily differences in baseline values via the randomized crossover design, an appropriately powered sample size, a consistent 2–7 day washout period, and by adding a control for the last consumed food the night prior to each visit. Our hypothesis was that participants may not have fully abided by the controlled meal the night prior to each visit, and or may have engaged in different levels of physical activity, thereby affecting energy balance, which may play a role in baseline differences in HOMA-IR between treatments (53).

Results from the current study indicate similar and consistent reductions in glycemic and insulinemic responses across doses typically used in FDA fiber food label testing (50g) as well as doses more typically experienced by consumers (30g). These findings suggest that substitution of RS4 in carbohydrate containing products may be a potentially feasible approach for helping to mitigate the increasing burden of non-communicable chronic diseases. In order to evaluate the previous conflicting RS4 evidence regarding glycemic and insulinemic responses to RS4 consumption, future research should 1) investigate differences between FDA approved and non-FDA approved testing methods and 2) investigate multiple doses of RS4 consumption utilizing FDA approved methods.

Figure 3.1. Consort Diagram

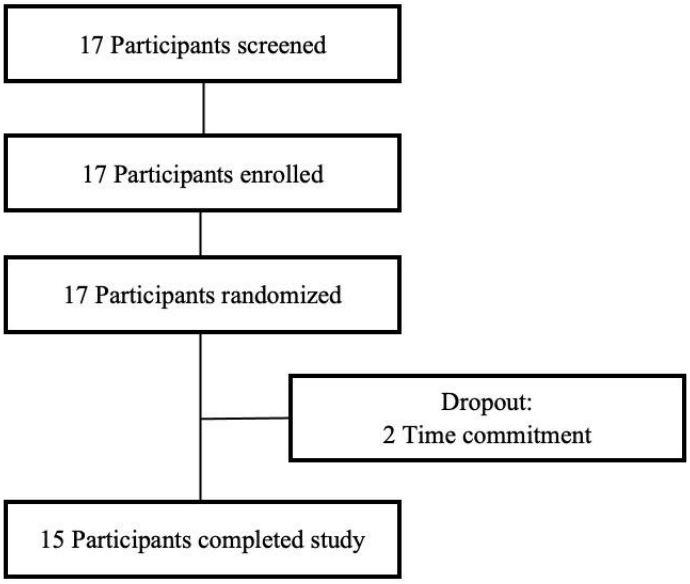


Figure 3.2. Postprandial glycemic and insulinemic responses to dextrose PWB and RS4 bars during the 2hr testing period. Data represent means and standard errors.

CAPTION: A) 50g Glucose iAUC response (mg/dL) B) 30g Glucose iAUC response (mg/dL) C) 50g Insulin response (μIU/mL) D) 30g Insulin response (μIU/mL). Treatments: 50Dex = 50g dextrose; 50PWB = 50g digestible carbohydrate of PWB; 50RS4 = 50g digestible carbohydrate of RS4; 30PWB = 30g digestible carbohydrate of PWB; 30RS4 = 30g digestible carbohydrate of PWB.

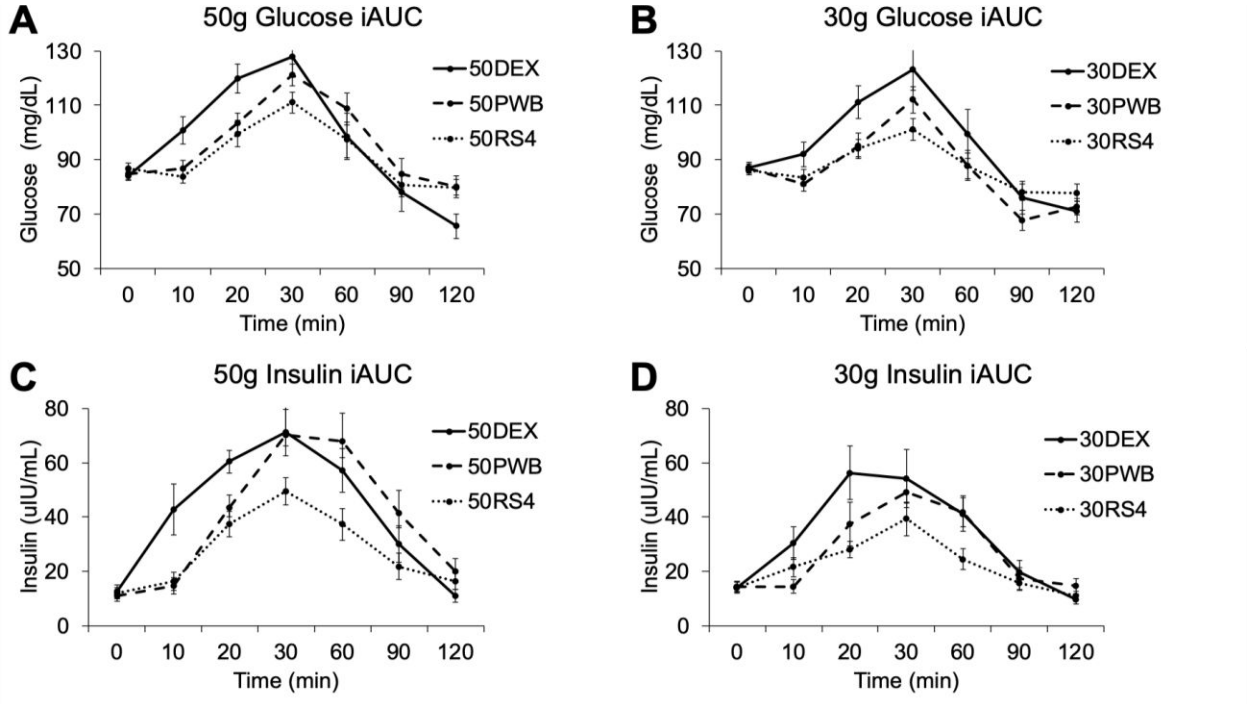


Table 3.1. Nutrient composition of PWB and RS4 bar treatments

Component	50PWB	50RS4	30PWB	30RS4
Ash (g)	1.6	1.8	0.6	0.6
Moisture (g)	13.7	18.0	4.9	6.4
Carbohydrate (g)	56.9	84.8	20.5	30.5
Digestible carbohydrate (g)	45.9	53.2	16.5	19.1
Dietary fiber (g)	11.0	31.6	4.0	11.4
Fat (g)	3.0	2.2	1.1	0.8
Protein (g)	9.1	6.4	3.2	2.3
Total calories (kcal)	246.9	258.6	88.8	93.0
Weight provided to participants (g)	91.7	106.4	55.0	63.8

Digestible carbohydrate was calculated as Carbohydrate (g) minus Dietary fiber (g).

Table 3.2. Baseline participant characteristics of individuals who completed the study. Results are reported as Mean ± SD.

	All (n=15)
Sex (Male;Female)	8;7
Age (yr)	26.1 ± 4.8
Height (cm)	174.0 ± 8.8
Weight (kg)	76.1 ± 16.8
BMI (kg/m^2)	24.9 ± 4.0
Body Fat (DXA, %)	23.6 ± 9.3
Fasting Glucose (mg/dL)	84.0 ± 5.77
Fasting Insulin (μIU/mL)	12.6 ± 9.29

DXA: Dual-energy X-ray Absorptiometry scan for body fat percentage.

Table 3.3. Summary of main effects of dose and treatment. Values are reported as mean estimates and 95% confidence interval.

		Treatment			Dose		
	Variable	DEX	PWB	RS4	30g	50g	*p*
Glucose	iAUC (mg/dL x 2hr)	1677* (1134, 2325)	1160* (721, 1703)	692 (365, 1123)	824 (480, 1260)	1508 (1020, 2093)	<0.001
	Peak (mg/dL)	132* (124, 142)	119* (111, 127)	108 (101, 115)	115 (108, 122)	123 (116, 132)	0.01
	Baseline-to-peak (mg/dL)	41.1* (29.2, 58.0)	27.8* (19.8, 39.1)	17.1 (12.2, 24.1)	22.0 (16.1, 30.0)	33.1 (24.0, 45.5)	0.005
	Time-to-peak (Minutes)	27.5 (23.3, 32.6)	34.3 (29.0, 40.5)	32.0 (27.0, 37.8)	32.9 (28.3, 38.3)	29.5 (25.2, 34.4)	0.12
Insulin	iAUC (µIU/mL x 2hr)	2642* (1993, 3382)	2574* (1934, 3305)	1419 (953, 1978)	1593 (1126, 2142)	2840 (2198, 3564)	<0.001
	Peak (µIU/mL)	67.3* (54.8, 82.7)	62.2* (50.6, 76.4)	45.6 (37.1, 56.0)	51.0 (41.9, 62.1)	65 (53.3, 79.2)	<0.001
	Baseline-to-peak (µIU/mL)	54.4* (43.8, 67.4)	50.6* (40.9, 62.5)	31.3 (25.3, 38.8)	35.8 (29.5, 43.4)	54.5 (44.6, 66.4)	<0.001
	Time-to-peak (Minutes)	27.6 (23.3, 32.7)	38.6 (32.7, 45.6)	33.0 (27.9, 39.0)	31.3 (27.2, 35.9)	34.3 (29.6, 39.8)	0.30
Indexes	HOMA-IR	2.50 (1.88, 3.31)	2.34 (1.77, 3.10)	2.40 (1.81, 3.18)	2.66 (2.03, 3.49)	2.18 (1.66, 2.87)	0.01

	Insulin:Glucose	1.95 (1.09, 3.49)	3.01 (1.69, 5.36)	2.75 (1.54, 4.89)	2.55 (1.48, 4.39)	2.50 (1.44, 4.34)	0.92

For treatment, comparisons with RS4 are reported where * = $p<0.05$. HOMA-IR was used to determine insulin resistance. Insulin-to-glucose ratio was used to determine impaired glucose tolerance. Equations for the indexes listed above can be found in the methods section.

Table 3.4. Summary of treatment means by dose. Values are reported as mean estimates and 95% confidence intervals.

		30g			50g		
	Variable	DEX	PWB	RS4	DEX	PWB	RS4
Glucose	iAUC (mg/dL x 2hr)	1432 (883, 2114)	693 (331, 1188)	481 (186, 914)	1940 (1249, 2783)	1746 (1109, 2528)	941 (501, 1519)
	Peak (mg/dL)	130 (120, 140)	114 (105, 123)	103 (95.5, 112)	135 (124, 147)	125 (114.7, 135)	112 (103.2, 121)
	Baseline-to-peak (mg/dL)	34.5 (23.1, 51.5)	22.0 (14.7, 32.8)	14.0 (9.3, 21.1)	49.0 (31.7, 75.8)	35.1 (23.0, 53.7)	21.0 (13.9, 31.6)
	Time-to-peak (Minutes)	31.8 (26.1, 38.7)	32.6 (26.8, 39.8)	34.3 (28.0, 42.1)	23.9 (19.2, 29.7)	36 (29.2, 44.4)	29.7 (24.2, 36.4)
Insulin	iAUC (µIU/mL x 2hr)	2184 (1519, 2970)	1789 (1193, 2506)	943 (519, 1493)	3143 (2317, 4095)	3501 (2625, 4502)	1993 (1347, 2764)
	Peak (µIU/mL)	59.1 (47.0, 74.3)	55.4 (44.0, 69.6)	40.6 (32.1, 51.2)	76.8 (60.8, 96.9)	69.8 (55.3, 88.2)	51.2 (40.5, 64.6)
	Baseline-to-peak (µIU/mL)	45.7 (35.4, 58.8)	40.7 (31.6, 52.4)	24.7 (19.1, 32.1)	64.7 (49.1, 85.4)	62.9 (48.1, 82.2)	39.7 (30.4, 52.9)
	Time-to-peak (Minutes)	29.5 (23.8, 36.7)	36.1 (29.1, 44.8)	28.7 (22.9, 35.9)	25.8 (20.3, 32.9)	41.3 (32.7, 52.0)	38 (30.1, 47.9)

Indexes	HOMA-IR	2.64 (1.94, 3.5)	2.70 (1.98, 3.67)	2.65 (1.95, 3.60)	2.36 (1.73, 3.23)	2.03 (1.50, 2.77)	2.17 (1.59, 2.96)
	Insulin:Glucose	1.98 (1.03, 3.80)	3.48 (1.81, 6.68)	2.41 (1.24, 4.69)	1.92 (0.95, 3.86)	2.60 (1.32, 5.15)	3.13 (1.61, 6.10)

HOMA-IR was used to determine insulin resistance. Insulin to glucose ratio was used to determine impaired glucose tolerance. Equations for the indexes listed above can be found in Steele, Maningat (20).

Chapter 4 - Postprandial glycemic and insulinemic responses to resistant starch type 4 (RS4) in healthy humans: A systematic literature review and meta-analysis of randomized controlled trials

High dietary fiber intake has been shown to be associated with a reduced risk of developing severe non-communicable chronic diseases such as cardiovascular diseases (CVD) and type 2 diabetes (4). Importantly, dietary fiber has been listed as a nutrient of concern for nearly two decades (33). Recently, the 2020–2025 Dietary Guidelines for Americans reported that more than 90% of women and 97% of men do not meet the recommended intakes of dietary fiber (34). In addition, the dietary guidelines state that the high prevalence of inadequate dietary fiber consumption aligns with inadequate consumption of fruits, vegetables, and whole grains in approximately 85% of adults (34). The new 2020 dietary guidelines include increasing dietary fiber intake as a "special consideration," and includes dietary fiber intake goals. For men, the recommended fiber intake is 34g, decreasing to 28g for men ages 51+, and for women intakes of 28g, decreasing to 22g for women aged 51+. These recommendations were all based on dietary reference intakes and are in accordance with previous guidelines reporting a recommended 14g of dietary fiber per 1000 calories of food intake (4, 7). Increasing dietary fiber intake appears to represent an opportunity for improving population health and reducing the risk of developing chronic diseases.

Emerging evidence indicates that resistant starch type 4 (RS4) consumption, a human manufactured starch that resists digestion, has beneficial effects on human health. The beneficial effects have been reported as reductions in postprandial glycemic and insulinemic responses (15, 17-25), total cholesterol (36), and body weight (36), among others. Recently, conflicting

evidence with respect to reductions in postprandial glycemic and insulinemic responses have been reported (16, 19, 20, 27). These conflicting results may be due to differences in testing methods that may elicit heterogeneous responses depending on the formulation of the tested products (20). Specifically, testing methods may vary with regard to whether digestible carbohydrates are matched across test and control conditions, per the FDA approved testing method. Some studies have not matched for digestible carbohydrate amounts across test conditions using non-FDA approved testing methods (26). To note, the two testing methods are described below:

1. The add-on approach adds the test ingredient on top of the control treatment. Thus, the starch ingredient is added on top of the control treatment recipe yielding a test treatment identical to the control treatment, albeit with the addition of the starch ingredient to the recipe.
2. The substitution approach substitutes the test ingredient, either partially or fully, in place of a carbohydrate containing ingredient in the control treatment. Thus, the substitution yields two treatments that differ only by the substitution of starch with the primary carbohydrate containing ingredient in the control treatment.

In a given experiment, if test and control conditions are matched for digestible carbohydrate then they follow the FDA approved testing method. In contrast, if test and control conditions are not matched for digestible carbohydrate then the experiment is investigating the non-FDA approved testing method. These various product formulations may have an impact on the glycemic and insulinemic responses following consumption of RS4 due to differences in digestible carbohydrate and caloric content. Comparing the different testing methods used in the current literature would advance the understanding of metabolic responses to RS4 consumption using

FDA approved testing methods compared with methods used to formulate products in the marketplace.

Therefore, the current systematic review and meta-analysis sought to determine the overall effects of RS4 consumption on postprandial glycemic and insulinemic responses compared to a control condition, including the highest quality evidence. Further, a sub-analysis investigating FDA approved testing methods compared with non-FDA approved substitution methods was conducted in order to address the potential differential effects these testing methods might have on glycemic and insulinemic responses.

Methods

Study Identification

This systematic review and meta-analysis was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRIMA) guidelines. Research databases including ProQuest Nursing & Allied Health, PubMed, Scopus, SpringerLink, and Web of Science Core Collection were searched through 4 February 2021. All databases were searched using the following search string: (("resistant starch") AND ("type 4" OR "type-4" OR "RS4") AND ("glycemic" OR "glucose" OR "postprandial glucose" OR "insulinemic" OR "insulin" OR "postprandial insulin")). Studies were included in this systematic review if they 1) investigated the effect of RS4 consumption, 2) were randomized controlled trials (RCTs), 3) were conducted using adult human subjects, 4) reported results for postprandial glucose and/or insulin, 5) were written in English, and 6) were published, peer-reviewed, full-text articles (no data from conference proceedings, abstracts, and textbooks). Studies were excluded if they did not meet all inclusion criteria.

Screening and Eligibility

The search process and results are depicted in Figure 4.1. All records from the databases (n=264) were imported into an excel file, in which one researcher identified and removed all duplicates. A total of 51 duplicate records were identified. During the next stage, titles and abstracts were independently screened by two of the authors. Following the title and abstract screening, a total of 185 records were excluded. Next, remaining records (n=28) underwent full-text screening by the same authors. Conflicts at each stage were discussed until agreement was reached for all included records. Full-text articles were imported into Endnote X9 (Clarivate) for review. Of those reviewed, 16 articles were excluded due to not investigating RS4 (n=9), study design other than an RCT (n=4), lacking postprandial glucose and/or insulin data (n=3). Therefore, 12 RCTs were included in the qualitative analysis.

Risk of Bias Assessment

A risk of bias assessment was conducted for all eligible studies and was completed independently by two authors using The Cochrane Risk of Bias tool for quality assessment of randomized controlled trials (54). The Cochrane Risk of Bias tool identifies potential sources of bias within studies based on random sequence generation (Selection Bias), allocation concealment (Selection Bias), blinding of participants and personnel (Performance Bias), and other criteria that may minimize bias within a study (Detection Bias, Attrition Bias, Reporting Bias, and Other Sources of Bias). Studies are reported as having a low, moderate, or high risk of bias depending on whether studies satisfied requirements for each source of bias. Disagreements among responses between authors were resolved through active discussion until an agreement was reached.

Data Extraction and Statistical Methods

Upon completion of screening, relevant data were extracted from each article by one of the authors and entered into a data table. To note, the formulation method is listed in Table 4.1 and is abbreviated to report which of the three methods was used. Specifically, the FDA approved add-on method is reported as "Add-on Matched", the FDA approved substitution method is reported as "Sub Matched", and the non-FDA approved substitution method is reported as "Sub Not Matched". Data collected included publication information (authors, year, RS4 source, etc.), number of participants, FDA approved method (Y; N), method (Add-on Matched; Sub Matched; Sub Not Matched), dose of RS4, method of blood collection (venous versus capillary), qualitative description of postprandial glucose and insulin outcomes (iAUC; peak; time-to-peak; baseline-to-peak), and postprandial venous glucose and insulin iAUC data (means, standard deviation [SD], standard error of the means [SEM], and the units of measurement). Peak refers to the maximum concentration, or the highest measured glucose/insulin value, across the postprandial assessment period, baseline-to-peak is the difference between maximum concentration and the baseline concentration. Studies that reported means for glucose iAUC and/or insulin iAUC were included in a meta-analysis. For studies that reported more than one RS4 condition and only one control condition, the results from highest RS4 dose and the control condition were included in the analysis. Of the 12 studies included for qualitative analysis, eight were included in the meta-analysis. An additional five studies were excluded from the quantitative analysis as one study reported medians, one study reported total AUCs only, and three studies did not report quantitative mean values.

All statistical analyses were performed using the software R (version 3.5.1) with package meta (41). Effect sizes were calculated using the inverse variance method. Given the significant

heterogeneity revealed by the Cochran Q test and Higgin I^2 statistic (reported in Figure 4.2 and Figure 4.3), as well as the methodological heterogeneity (e.g. different doses, different sources), we proceeded with a random effects model to minimize bias (55). We performed a standard mean difference (SMD) analysis to account for different units of iAUC values. The SMD was calculated using Hedge's g. A negative value indicates that the results favor RS4 while a positive value indicates that results favor the control.

The overall analysis evaluated the difference in glucose iAUC and insulin iAUC between RS4 and control conditions, regardless of testing method. Subgroup analysis investigated whether differences in glucose iAUC and insulin iAUC differed as a function of testing method (FDA approved versus non-FDA approved). For the subgroup analysis, the comparison was considered statistically significant if the confidence interval did not include 0. In addition, sensitivity analyses were performed to determine if the FDA approved add-on method was influencing the results of the analyses. The sensitivity analysis was performed by calculating the SMD after excluding studies that utilized the add-on FDA approved method. The treatment effect was considered significant if $p<0.05$. To note in the reporting of results, counts of qualitative data points (total datapoints for a given outcome) may appear larger than the 12 included studies as multiple studies reported multiple data points for a given outcome; for example, some studies reported on capillary and venous samples, resulting in two data points for the single study. Therefore, the total number of datapoints is larger in comparison to the total number of studies.

Results

Description of Selected Trials

Table 4.1 depicts the characteristics of the included studies. The database searches and screening process yielded a total of 12 included RCTs, with five studies using an FDA approved method (15, 17, 19, 20, 27), and eight studies using the substitution method not approved by the FDA (16, 18, 20-25). Note that one study included both an FDA approved treatment arm and a non-FDA approved treatment arm (20). The source of RS4 differed among studies: wheat (15, 16, 18, 20), maize (22, 23, 25), potato (17, 19, 21, 27), and tapioca (24). Of those that used an FDA approved method matched for digestible carbohydrate, two studies used an add on method (20, 27) and two studies used a substitution method (15, 17). All non-FDA approved methods used a substitution method without matching for digestible carbohydrate. The doses for the FDA approved methods ranged from 20−30 g of fiber from RS4, while the doses for the non-FDA approved methods ranged from 5−38 g of fiber from RS4.

Study Risk of Bias

As evaluated using the Cochrane Risk of Bias tool, a total of eight studies were scored as low risk of bias, 2 studies were scored as moderate risk of bias, and three studies had an unclear risk of bias (Table 4.2). The primary reasons for the two studies with moderate risk of bias designations were unclear selection bias, detection bias, and other sources of bias related to author affiliations and funding (17, 22). Additionally, one study had unclear selection bias related to randomized sequence generation (22) and the second lacked information related to attrition bias (17). The two studies that were scored as unclear risk of bias included combinations of unclear selection bias, performance bias, detection bias, and attrition bias (18, 25). Additionally, one of the two unclear risk of bias studies included other sources of bias concerns

related to author affiliations and funding (25). All studies included were randomized control trials which lends to a lower overall risk of bias among the included studies.

Meta-Analysis for Glucose and Insulin iAUC

Glucose iAUC was significantly lower following consumption of RS4 compared to control conditions (SMD: -1.93; 95%CI: -2.92, -0.94; z = -3.82; $p < 0.001$), as depicted in Figure 4.2. The subgroup analysis revealed that glucose iAUC was significantly lower following consumption of RS4 compared to control conditions for FDA approved methods (SMD: -1.33; 95%CI: -2.42, -0.24) and non-FDA approved methods (SMD: -2.49; 95%CI: -4.15, -0.82). The SMDs for FDA and non-FDA approved methods were not significantly different, $p = 0.25$. The sensitivity analysis revealed that when Du, Wu (27) and Steele, Maningat (20), studies that used the FDA approved add-on method, were excluded from the analysis, glucose iAUC remained significantly lower following RS4 consumption as compared to the control (SMD: -2.51; 95%CI: -3.63, -1.39; z = -4.38; $p < 0.001$), consistent with the overall analysis and subgroup analysis. While the overall conclusion remained the same, the effect size for the FDA approved methods was larger in the sensitivity analysis when the studies utilizing the FDA approved add-on method were removed (SMD: -2.58; 95%CI: -4.76, -0.41) compared to the original analysis with both FDA approved methods included (SMD: -1.33; 95%CI: -2.42, -0.24).

Insulin iAUC was significantly lower following consumption of RS4 compared to control conditions (SMD: -2.97; 95%CI: -4.64, -1.30; z = -3.49; $p < 0.001$), as depicted in Figure 4.3. The subgroup analysis revealed that insulin iAUC was not significantly different between RS4 conditions compared to control conditions for FDA approved methods (SMD: -2.06; 95%CI: -4.49, 0.36). However, insulin iAUC was significantly lower following consumption of RS4 compared to a control condition for non-FDA approved methods (SMD: -3.88; 95%CI: -4.60, -

3.16). The SMDs for FDA and non-FDA approved methods were not significantly different, p = 0.16. The sensitivity analysis revealed that insulin iAUC remained significantly lower following RS4 consumption compared to control conditions (SMD: -4.01; 95%CI: -4.50, -3.53; z = -16.10; $p < 0.001$), consistent with the overall analysis. Inconsistent with the subgroup analysis, the sensitivity analysis revealed that insulin iAUC was significantly different between RS4 conditions compared to control conditions for FDA approved and non-FDA approved methods. In addition, the effect size for the FDA approved methods was larger in the sensitivity analysis when the studies utilizing the add-on method were removed (SMD: -4.26; 95%CI: -4.96, -3.56) compared to the original analysis with all FDA approved methods included (SMD: -2.06; 95%CI: -4.49, 0.36).

Qualitative Analysis of Glucose and Insulin Outcome Measures

A qualitative description of postprandial glucose and insulin outcomes for each study is outlined in Table 4.1 and a summary of the results are described in this section. Of the 13 data points reported for glucose iAUC, eight studies (two FDA approved (15, 17) and six non-FDA approved (18, 21-25)) found that glucose iAUC was significantly lower following consumption of the RS4 conditions compared to the control conditions, while five studies (four FDA approved (17, 19, 20, 27) and one non-FDA approved (20)) found no significant difference in glucose iAUC between RS4 and control conditions. Four studies reported glucose iAUC from capillary and venous samples; two studies found that glucose iAUC was lower regardless of blood collection method (21, 22), one study found that glucose iAUC was lower for the venous sample and not the capillary sample (23), and one study found that glucose iAUC was lower for the capillary sample and not the venous sample (17). Of the nine data points for insulin iAUC, seven studies (two FDA approved (15, 17) and six non-FDA approved (16, 20-24)) found that insulin

iAUC was significantly lower following consumption of the RS4 conditions compared to the control conditions, while two studies (two FDA approved (20, 27) and no non-FDA approved) found no significant difference in insulin iAUC between RS4 and control conditions.

Of ten data points reported for glucose peak, six studies (two FDA approved (15, 17) and four non-FDA approved (21-24)) found that glucose peak was significantly lower following consumption of the RS4 conditions compared to the control conditions, while five studies (two FDA approved (20, 27) and three non-FDA approved (20, 23, 24)) found no significant difference in glucose peak between RS4 and control conditions. Of three data points reported for glucose baseline-to-peak, one study (one FDA approved (15) and no non-FDA approved) found that glucose baseline-to-peak was significantly lower following consumption of the RS4 conditions compared to the control conditions, while one studies (one FDA approved (20) and one non-FDA approved (20)) found no significant difference in glucose baseline-to-peak between RS4 and control conditions. Of four data points reported for glucose time-to-peak, all studies (two FDA approved (20, 27) and two non-FDA approved (20, 24)) found no significant difference in glucose time-to-peak between RS4 and control conditions.

Of nine data points reported for insulin peak, four studies (one FDA approved (17) and three non-FDA approved (20-22)) found that insulin peak was significantly lower following consumption of the RS4 conditions compared to the control conditions, while four studies (two FDA approved (20, 27) and two non-FDA approved (23, 24)) found no significant difference in insulin peak between RS4 and control conditions. One FDA approved study found that insulin peak was significantly higher following consumption of the RS4 condition compared to the control condition (15). Of three data points reported for insulin baseline-peak, two studies (one FDA approved (15) and one non-FDA approved (20)) found that insulin baseline-to-peak was

significantly lower following consumption of the RS4 conditions compared to the control conditions, while one study (one FDA approved (20) and no non-FDA approved) found no significant difference in insulin baseline-to-peak between RS4 and control conditions. Of four data points reported for insulin time-to-peak, all studies (two FDA approved (20, 27) and two non-FDA approved (20, 24)) found no significant difference in insulin time-to-peak between RS4 and control conditions.

Discussion

The primary aim of the current systematic review and meta-analysis was to determine the overall effect of RS4 consumption on postprandial glycemic and insulinemic responses as compared with a control. The findings of the meta-analysis indicated that RS4 elicits significantly lower glucose and insulin iAUC responses compared to control conditions. The subgroup analysis, parsing apart methodological differences, indicated that these responses may depend on whether an FDA approved or non-FDA approved testing method was used. Specifically, the subgroup analysis reported that glucose iAUC remained significantly lower across both FDA and non-FDA approved testing methods following consumption of RS4 compared to control conditions. However, insulin iAUC was not significantly lower for FDA approved testing methods, whereas insulin iAUC was significantly lower for non-FDA approved testing methods following RS4 consumption compared with control conditions. Thus, glucose iAUC results were consistent across the overall analysis and subgroup analysis for FDA approved and non-FDA approved testing methods, whereas insulin iAUC was primarily reduced following RS4 consumption for studies using non-FDA approved substitution methods.

The subgroup analysis must be interpreted with caution given that the sensitivity analysis yielded different outcomes following the removal of the two studies investigating the FDA

approved add-on method. When the two studies utilizing the FDA approved add-on method were removed, the effect size for glucose iAUC was strengthened. In addition, insulin iAUC was significantly lower for RS4 conditions compared to the control conditions. The differences in glycemic and insulinemic outcomes between the two FDA approved testing methods requires further investigation, although the current data suggests that beneficial glycemic and insulinemic responses may only be observed when formulating products using the FDA approved substitution method. As seen in Figure 4.2, glucose iAUC remained significantly lower for both FDA approved and non-FDA approved testing methods.

The impact of the studies utilizing the FDA approved add-on method on the subgroup analysis can be seen in Figure 4.3 when comparing the four studies investigating FDA approved testing methods (15, 17, 20, 27). Specifically, Du, Wu (27) and Steele, Maningat (20), the studies that utilized the FDA approved add-on method, reported insulin iAUC values that were similar between RS4 and control conditions. In contrast, Al-Tamimi, Seib (15) and Gourineni, Stewart (17), the studies that utilized the FDA approved substitution method, reported reduced insulin iAUC responses following RS4 consumption compared with control conditions. This distinction suggests that the FDA approved add-on method may not identify beneficial glycemic and insulinemic responses following RS4 consumption whereas the FDA approved substitution method appears to result in beneficial glycemic and insulinemic responses following RS4 consumption.

The intriguing results from the overall and subgroup analyses require additional context with respect to the current FDA approved fiber food label testing standards. The FDA approved add-on method may allow for a confounding variable of multiple carbohydrate sources. However, the FDA approved substitution method only differs from the non-FDA approved

substitution method by the requirement of matched digestible carbohydrate amounts between testing conditions. This matching aspect is an essential element of FDA approved methods as it eliminates the confounding effects of markedly different amounts of digestible carbohydrate on glycemic and insulinemic responses (26). Fundamentally, matching digestible carbohydrate amounts is conservative and yields results reflecting the independent impact of the test ingredient.

Regarding the non-FDA approved testing method, the marketplace substitution method substitutes the test ingredient in place of a carbohydrate containing ingredient, although no matching of digestible carbohydrate is implemented. This method is used by food manufacturers for packaging of products for the marketplace. In this context, the added fiber is primarily used in place of other carbohydrate ingredients to increase fiber in food products, thus reducing the total carbohydrate and caloric content of the product. Therefore, the non-FDA approved substitution method is intended to test products that may better reflect how products are manufactured, packaged, and purchased by consumers. There is an important distinction between the way fibers are tested for FDA fiber food label approval— a conservative method to test the independent effect of the fiber— and the non-FDA approved substitution method, a method where products may have increased benefit from reduced digestible carbohydrate and calorie content. The results from the current meta-analysis suggest that glycemic responses are similar between FDA approved and non-FDA approved testing methods. However, insulin responses appear to depend on the method of testing for beneficial reductions in insulinemic responses following RS4 consumption as compared with a control. Both the FDA approved, and non-FDA approved testing methods have important uses as well as strengths and limitations, although the

FDA approved testing methods appears more appropriate to distinguish the independent effect of a fiber ingredient.

The collective results of the included RCTs indicate that approximately 65% of studies observed a significantly lower RS4 glucose peak and 50% observed a significantly lower RS4 insulin peak compared to control conditions. These percentages do not take into account the different testing methods; thus, these results should be interpreted with caution. However, the glucose and insulin peak results suggest that further research is warranted across all testing methods in order to identify any potential benefits associated with attenuations in glucose and insulin peaks, which are considered to be important markers of glycemic control (56). Glucose baseline-to-peak was significantly lower following RS4 consumption in 33% of studies whereas insulin baseline-to-peak was significantly lower following RS4 consumption in 66% of reported studies. These results suggest a potential reduction in the magnitude of responses of glucose and insulin following RS4 consumption. Recent research suggests that a reduction in magnitude of postprandial glucose response may be related to a beneficial response of diabetes related risk factors (57), although further research is necessary to determine any definitive conclusions. Lastly, no included studies reported differences between RS4 and control conditions for glucose or insulin time-to-peak, markers of diabetes risk (58). Although these results are limited in nature, with less than five studies reporting time-to-peak and baseline-to-peak values across the 12 included studies, the results warrant further research to identify any potentially beneficial metabolic outcomes related to glycemic and insulinemic responses following RS4 consumption.

As with any study, there are some limitations that should be considered when interpreting this systematic review and meta-analysis. For some of the studies included in this systematic review, there was an uncertain risk of bias (Table 4.2). This is likely due to these studies being

published either during, or prior to, 2010 when reporting requirements indicated in the CONSORT Statement were limited. However, all included studies inherently maintained a lower level of bias given the randomized controlled trial inclusion criteria. In addition, approximately half of the appraised studies contained small sample sizes (n<15) which may not represent the general population. Another limitation among the included studies was that some studies used multiple doses for experimental conditions with only one dose for the control condition. To account for this issue, we extracted data for the highest dose condition in order to avoid the inappropriate reporting of the control condition for two comparisons. In addition, the sensitivity analysis revealed a difference in statistical significance upon removal of the FDA approved addition studies as compared with the full analysis. Therefore, there may be differences in postprandial glycemic and insulinemic responses following consumption of RS4 between the FDA approved addition and substitution methods. Lastly, there were limited available data that met the inclusion criteria for this systematic review and meta-analysis which leaves a few potential gaps in our understanding of additional potential confounders (source of RS4; cooked vs uncooked conditions). It is well established that factors such as heating and the source of RS4 can have an impact on the amount of RS4 in a given product (59). In addition, the inherent differences between the physical and behavioral characteristics of the different sources of RS4 can play a role in the digestibility of RS4 (17). Thus, the limited available data prohibits investigation of these factors.

This systematic review and meta-analysis found that RS4 consumption reduces postprandial glucose and insulin iAUC compared with control conditions, and that the reductions are not significantly different based on the FDA approved or non-FDA approved testing method. The Dietary Guidelines for Americans have consistently shown underconsumption of dietary

fiber among Americans and have included dietary fiber is a dietary component of concern since 2005. Additionally, reductions in postprandial glycemic and insulinemic responses have consistently been shown to be associated with reductions in risk for CVD and type 2 diabetes (28). While non-communicable chronic diseases are complex and will require multi-faceted solutions, the inclusion of RS4 in marketplace products may be a feasible approach for increasing fiber consumption for helping to mitigate the increasing burden of non-communicable chronic diseases.

Figure 4.1. Flow chart of literature search and selection process across 5 relevant databases. RS4: resistant starch type 4; RCT: randomized controlled trial.

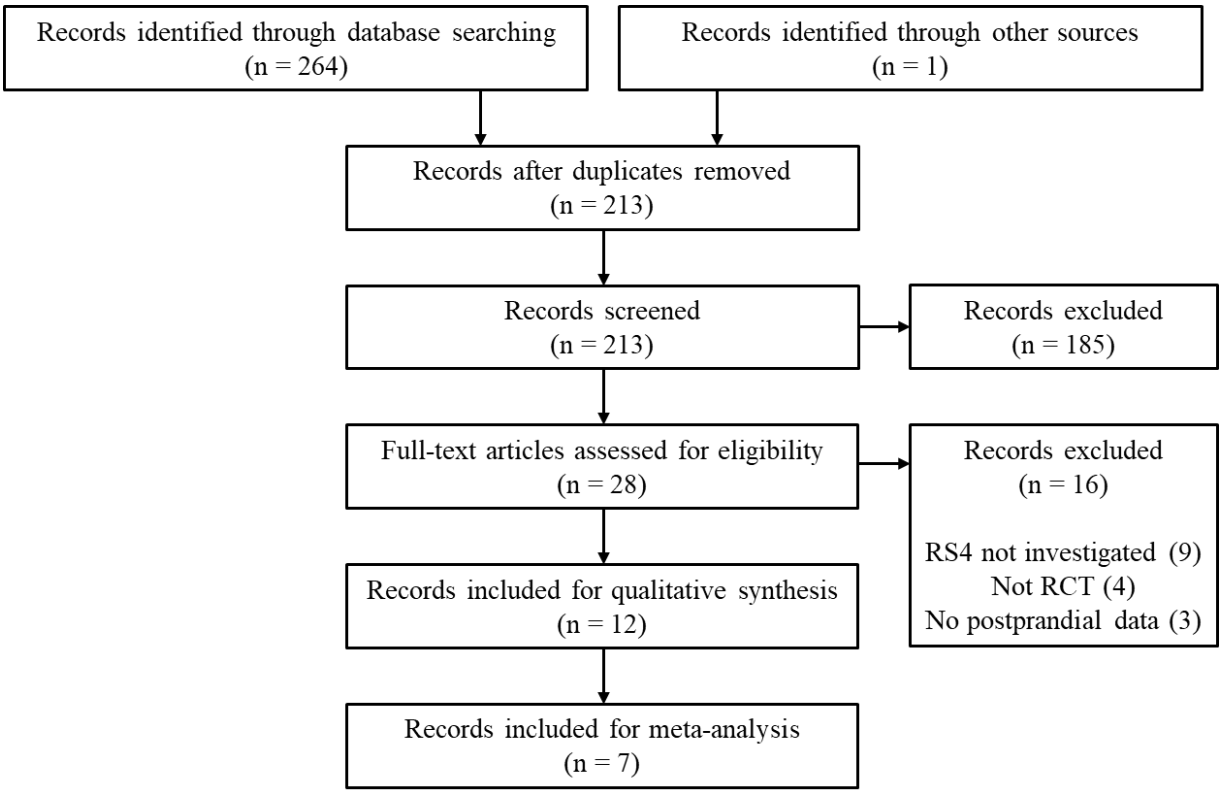
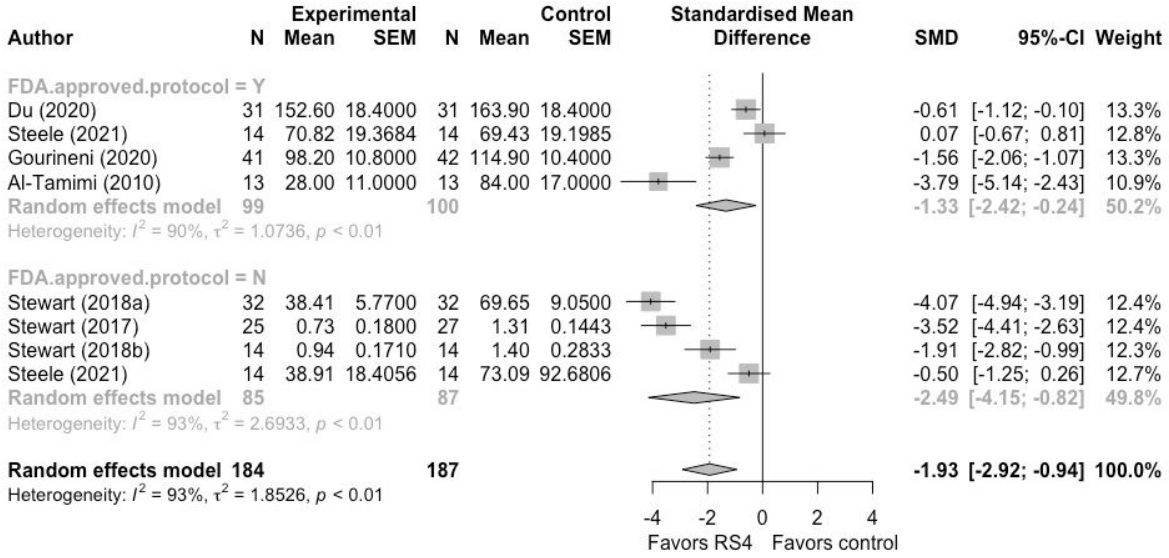
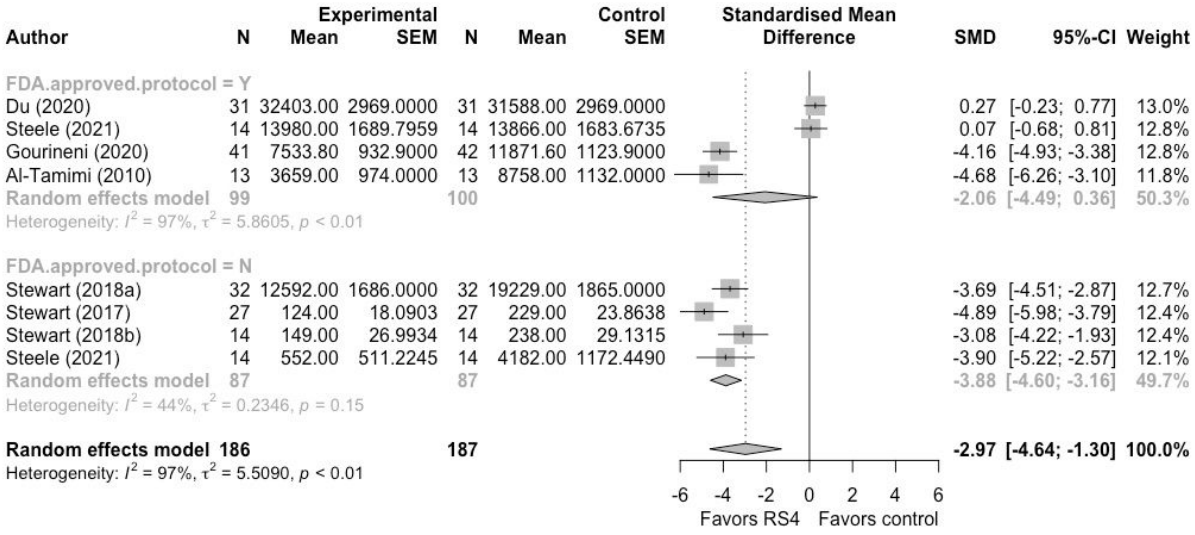


Figure 4.2. Forest plot illustrating the standard mean difference (SMD) between RS4 and control conditions for venous glucose iAUC.



The overall effect is reported in black, while the subgroup analysis effects are reported in gray. The two subgroups are designated as Y (yes) for FDA approved testing methods and N (no) for non-FDA approved testing methods. The FDA approved testing method subgroup contains both FDA approved addition and substitution testing methods. SEM = standard error of the mean.

Figure 4.3. Forest plot illustrating the standard mean difference (SMD) between RS4 and control conditions for venous insulin iAUC.



The overall effect is reported in black, while the subgroup analysis effects are reported in gray. SEM = standard error of the mean.

Table 4.1. Characteristics of included studies. All reported findings are obtained from venous samples unless otherwise noted in parentheses.

Study	Brand	RS4 Source	Control Source	Type of Food	AOAC Method	Cooked or Raw	Testing Method	RS4 Dose (g of fiber)	Length of Protocol	Sample Size (n)	Findings for RS4 versus Control Conditions
FDA approved method											
Al-Tamimi, Seib (15)	Fibersym®RW (MGP Ingredients, Inc.; Atchison, KS)	Wheat	Puffed Wheat	Bar	991.43	Heated to 85˚C	Sub Matched	20	2hrs	13	↓ Glucose iAUC ↓ Insulin iAUC ↓ Glucose peak ↑ Insulin peak ↓ Glucose baseline-to-peak ↓ Insulin baseline-to-peak
Du, Wu (27)	VersaFibe™ 1490 (Ingredion Incorporated; Bridgewater, NJ)	Potato	Rice Krispies Cereal	Cereal bar	991.43	Heated to 60˚C	Add-on Matched	20	2hrs	31	↔ Glucose iAUC ↔ Insulin iAUC ↔ Glucose peak ↔ Insulin peak ↔ Glucose time-to-peak ↔ Insulin time-to-peak
Gourineni, Stewart (17)	VersaFibe™ 1490 (Ingredion Incorporated; Bridgewater, NJ)	Potato	Puffed Wheat	Bar	991.43	Heated to 85˚C	Sub Matched	30	2hrs	41	↔ Glucose iAUC ↓ Glucose iAUC (capillary) ↓ Insulin iAUC ↓ Glucose peak ↓ Glucose peak (capillary) ↓ Insulin peak
Haub, Louk (19)	PenFibe (Penford Food Ingredients; Centennial, CO)	Potato	Dextrose	Drink	NR	Raw	Add-on Matched	30	2hrs	10	↔ Glucose iAUC (capillary)

Steele, Maningat (20)	Fibersym®RW (MGP Ingredients, Inc.; Atchison, KS)	Wheat	Native Wheat Starch	Cracker	991.43	Heated to 85˚C	Add-on Matched	21	2hrs	14	↔ Glucose iAUC ↔ Insulin iAUC ↔Glucose peak ↔ Insulin peak ↔ Glucose time-to-peak ↔ Insulin time-to-peak ↔ Glucose baseline-to-peak ↔ Insulin baseline-to-peak
Non – FDA approved method											
Emilien, Hsu (16)	Fibersym®RW (MGP Ingredients, Inc.; Atchison, KS)	Wheat	All-purpose Flour	Muffin	NR	NR	Sub Not matched	26	4hrs	27	↔ Glucose AUC ↓ Insulin AUC
Haub, Hubach (18)	Fibersym®RW (MGP Ingredients, Inc.; Atchison, KS)	Wheat	Dextrose	Water + starch	NR	Raw	Sub Not matched	30	2hrs	11	↓ Glucose iAUC (capillary)
Mah, Garcia-Campayo (24)	Actistar 75330 (Cargill, Inc.; Minneapolis, MN)	Tapioca	Standard Corn Starch	Bar	991.43	Heated to 204˚C	Sub Not matched	32	2hrs	21	↓ Glucose iAUC (capillary) ↓ Insulin iAUC ↔ Glucose peak (capillary) ↔ Insulin peak ↔ Glucose time-to-peak (capillary) ↔ Insulin time-to-peak

Steele, Maningat (20)	Fibersym®RW (MGP Ingredients, Inc.; Atchison, KS)	Wheat	Native Wheat Starch	Cracker	991.43	Heated to 85˚C	Sub Not matched	21	2hrs	14	↔ Glucose iAUC ↓ Insulin iAUC ↔Glucose peak ↓ Insulin peak ↔ Glucose time-to-peak ↔ Insulin time-to-peak ↔ Glucose baseline-to-peak ↓ Insulin baseline-to-peak
Stewart and Zimmer (23)	VersaFibe™ 2470 (Ingredion Incorporated; Bridgewater, NJ)	Maize	Not Stated	Muffin	2009.01	Heated (NR)	Sub Not matched	11.6	2hrs	28	↓ Glucose iAUC ↔ Glucose iAUC (capillary) ↓ Insulin iAUC ↓ Glucose peak ↔ Glucose peak (capillary) ↔ Insulin peak
Stewart and Zimmer (21)	VersaFibe™ 1490 (Ingredion Incorporated; Bridgewater, NJ)	Potato	Not Stated	Cookie	991.43	NR	Sub Not matched	25	2hrs	28	↓ Glucose iAUC ↓ Glucose iAUC (capillary) ↓ Insulin iAUC ↓ Glucose peak ↓ Glucose peak (capillary) ↓ Insulin peak
Stewart, Wilcox (22)	VersaFibe™ 2470 (Ingredion Incorporated; Bridgewater, NJ)	Maize	Flour (High Gluten & Pastry)	Scone	2009.01	Heated (NR)	Sub Not matched	16.5	3hrs	35	↓ Glucose iAUC ↓ Glucose iAUC (capillary) ↓ Insulin iAUC ↓ Glucose peak ↓ Glucose peak (capillary) ↓ Insulin peak

Tachibe, Nishibata (25)	Hydroxypropyl Distarch Phosphate (Matsutani Chemical Industry Co., Ltd.; Itami, Japan)	Maize	Native Tapioca Starch	Cookie	2001.03	NR	Sub Not matched	5	3hrs	10	↓ Glucose iAUC (capillary)

RS4: Resistant starch type 4; NR: Not Reported; Sub = substitution; iAUC: incremental area under the curve; Peak: maximum concentration (highest observed value); ↓: significantly lower than the control condition; ↑: significantly higher than the control condition; ↔: no significant difference between the RS4 and control groups.

Table 4.2. Risk of bias assessment for the 12 included studies. Type of bias is indicated in parentheses.

Study	Risk of bias	Random sequence generation (Selection)	Allocation concealment (Selection)	Blinding of participants and personnel (Performance)	Blinding of outcomes (Detection)	Complete data (Attrition)	Non-selective reporting (Reporting)	Other sources of bias
FDA approved method								
Al-Tamimi, Seib (15)	Low	+	?	+	?	+	+	–
Du, Wu (27)	Low	?	?	+	+	+	+	-
Gourineni, Stewart (17)	Moderate	+	?	+	?	-	+	-
Haub, Louk (19)	Low	+	?	+	?	+	+	+
Steele, Maningat (20)	Low	+	?	+	?	+	+	-
Non – FDA approved method								
Emilien, Hsu (16)	Low	?	?	+	?	+	+	?

Haub, Hubach (18)	Unclear	+	?	?	?	?	+	+
Mah, Garcia-Campayo (24)	Low	+	+	+	+	+	+	–
Steele, Maningat (20)	As described above							
Stewart and Zimmer (23)	Low	+	+	+	?	+	+	–
Stewart and Zimmer (21)	Low	+	?	+	?	+	+	–
Stewart, Wilcox (22)	Moderate	?	?	+	?	+	+	–
Tachibe, Nishibata (25)	Unclear	?	?	?	?	+	+	–

Chapter 5 - Conclusions

Resistant starch type 4 has been shown to reduce the risk of developing non-communicable chronic diseases (31, 60). Based on the available evidence, RS4 is an ideal candidate for investigation of feasible substitution interventions following nearly two decades of underconsumption (33) and the recent 2020–2025 Dietary Guidelines for American's reporting that 90% of Americans are not meeting the recommended intake levels (7). A recent review (61) suggests that mean daily fiber intake is approximately 16g of dietary fiber, merely half of the recommended intake for a middle-aged adult male according to the recent dietary guidelines for Americans (7). The present dissertation sought to investigate the effects of acute RS4 consumption on postprandial glycemic and insulinemic responses using FDA approved and non-FDA approved testing methods, and to summarize the highest quality of evidence investigating the acute effects of RS4 consumption on postprandial glycemic and insulinemic responses. Collectively, the series of studies 1) indicated differential glycemic and insulinemic responses using an FDA approved and non-FDA approved testing method following consumption of RS4 and NWS crackers; 2) identified similar and consistent reductions in postprandial glycemic and insulinemic responses following RS4 consumption across two doses of digestible carbohydrate formulated using an FDA approved testing method; and 3) identified that across multiple RCTs, RS4 consistently reduced glycemic and insulinemic responses compared with control treatments, although these results may depend on the testing method used.

In Chapter 2, we report results from a randomized controlled cross-over trial comparing the postprandial glycemic and insulinemic responses to three cracker treatments using two testing methods, the FDA approved add-on method and the non-FDA approved marketplace substitution method. We investigated five different treatments, three of which utilized the FDA

approved testing method matched for digestible carbohydrate (50Dex, 50NWS, and 50RS4), and two (35NWS and 35RS4) utilizing the marketplace substitution method. There were no significant differences in glucose or insulin iAUC between the 50g cracker conditions, and no differences in glucose iAUC between 35g cracker conditions. However, insulin iAUC was significantly lower following consumption of 35RS4 compared to 35NWS. These findings suggest that the addition of RS4 to formulated food products may not attenuate the glycemic response when 50g of digestible carbohydrates are added on top of NWS. However, the reduced insulinemic responses following consumption of 35RS4 crackers suggests the need for additional research investigating FDA approved and non-FDA approved testing methods, in addition to insulinemic responses of products manufactured, packaged, and purchased by consumers.

In Chapter 3, our focus shifted to the sole use of the FDA approved substitution methods and focused on how the dose of digestible carbohydrates affected postprandial glycemic and insulinemic responses following RS4 consumption. As such, we conducted a randomized controlled cross-over trial investigating acute postprandial glycemic and insulinemic responses to RS4 as compared with PWB and DEX, using two doses of digestible carbohydrate in accordance with the FDA approved substitution method. We investigated three treatments (DEX, PWB, RS4) across two doses (50g and 30g) of digestible carbohydrate yielding six total treatments grouped by dose (50Dex, 50PWB, 50RS4 and 30DEX, 30PWB, and 30RS4). There was a main effect of dose and treatment on glucose and insulin iAUC such that RS4 was lower than PWB and DEX conditions at both the 50g and 30g doses. These findings suggest that reductions in glucose and insulin iAUC are consistent between FDA approved and non-FDA approved testing methods regardless of the consumption of a 50g or 30g dose of RS4.

Lastly, in Chapter 4 we conducted a systematic review and meta-analysis of RCTs to determine the impact of acute RS4 consumption on postprandial glycemic and insulinemic responses. Within this study, we performed a subgroup analysis investigating FDA approved testing methods (add-on or substitution with matched digestible carbohydrate) as compared with non-FDA approved testing methods (marketplace substitution method). Our results suggested that RS4 consumption elicits reduced postprandial glucose and insulin iAUC responses compared with control treatments. The subgroup analysis, although limited by the number of studies, suggests that both the FDA approved testing methods and non-FDA approved testing method indicated a reduced glucose iAUC response following RS4 consumption. However, the FDA approved testing method did not result in reduced insulin iAUC responses following RS4 consumption, whereas the non-FDA approved testing method did. These findings suggest that acute RS4 consumption elicits reduced postprandial glycemic and insulinemic responses when using testing methods that reflect products that are manufactured, packaged, and purchased by consumers. Based on our additional sensitivity analysis, the data suggest that the FDA approved add-on method may not identify beneficial glycemic and insulinemic responses following RS4 consumption whereas the FDA approved substitution method appears to report physiological benefits to human health following RS4 consumption.

In conclusion, this series of studies enhances our understanding of the effects of acute RS4 consumption on postprandial glycemic and insulinemic responses. Testing methods appear to be a primary factor involved in the magnitude of postprandial glucose and insulin responses. Although the available evidence is scarce, the consistency of postprandial glycemic and insulinemic responses following RS4 consumption indicate the potential for RS4 to benefit human health. The research herein indicates that additional research is necessary to elucidate the

differences between the two FDA approved testing methods (add-on vs substitution) and studies investigating other physiological effects that indicate benefits to human health in order to identify other potential benefits of RS4 consumption. Overall, the non-FDA approved substitution method, which is primarily how products are manufactured, packaged, and purchased by consumers, reports consistent beneficial glycemic and insulinemic effects. Products formulated with RS4 may provide an opportunity for improved metabolic responses as compared with similar products containing higher amounts of digestible carbohydrate and calories. The results herein suggest the need for additional studies investigating comparisons similar to the comparisons investigated in Chapter 2 - , between FDA approved and non-FDA approved testing methods. The results from the meta-analysis show that the effects following the substitution method, regardless of FDA approved or non-FDA approved approaches, are very consistent. Therefore, additional research in needed comparing FDA approved testing methods and non-FDA approved substitution methods to understand how the different formulations effect a product's acute and long-term effect on health outcomes. Lastly, future research should investigate the effects of increasing fiber intake via RS4 consumption using a long-term intervention approach to determine the potential to mitigate the increasing prevalence of non-communicable diseases.

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