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Preliminary study on the effects of *Akkermensia muciniphilia* on Akt phosphorylation in diabetic pig models

By

Brian Ko

Abstract

The onset of type 2 diabetes has been shown to affect the Akt signaling pathway, responsible for modulating cellular metabolism. The probiotic *Akkermensia muciniphilia* has been recently shown to have an anti-inflammatory, anti-diabetic effect on animals inoculated with it, particularly within the Akt signaling pathway. This preliminary study utilized a two-factor experimental design with streptozotocin-induced diabetes and the presence of *Akkermensia muciniphila* in pig models in order to elucidate the probiotic's mechanism of action on the disease. Following analysis of insulin-sensitive tissues of muscle, pancreas, and liver, there was observed a mixed response to the presence of the probiotic and diabetes separately and together in the three tissues. This leaves open the possibility of the use of the probiotic as a preventative measure for diabetes.

Introduction

The world faces an obesity epidemic as processed foods take over our diets and disrupt our natural nutritional habits. Approximately 10% of the global population is obese, and the trend is positive for the foreseeable future (Friedrich, 2017). Along with the obesity epidemic comes the health risks associated with it such as high blood pressure and notably, type 2 diabetes. The cost for type 2 diabetes management is high. In the United States alone, the aggregate cost of diagnosed diabetes in 2017 was $327 billion. Out of the $327 billion, $237 billion went to direct medical costs and $90 billion was lost in decreased productivity (Yang, 2018)

There is an obvious need for some sort of treatment for such an epidemic disease. By administering the probiotic *Akkermensia muciniphilia* to pigs and then inducing diabetes in them, this study will be a step towards finding a possible preventative measure for type 2 diabetes. At the very worst, a better understanding of the effect of the presence of a probiotic on insulin resistance can be obtained.

Type 2 diabetes

In a healthy individual, the pancreatic islet beta cells that produce insulin for the body are able to increase insulin output to compensate for reduced insulin sensitivity, thus maintaining overall glucose tolerance. In individuals with type 2 diabetes, the beta cells are unable to produce enough insulin to compensate for that decreased insulin sensitivity.

Beta cells are highly plastic in their ability to regulate insulin release (Kahn, 2006). In healthy individuals, this insulin release is dependent on the nature, quantity, and route of administration of the stimulus, as well the prevailing glucose concentration. Insulin sensitivity

modulates beta cell function. For healthy individuals, a feedback loop exists between insulin-sensitive tissues and the beta cells, with an increased insulin supply in response to demand from liver, muscle, and adipose tissues. A failure in this feedback loop alters overall glucose tolerance and leads to diabetes.

Changes to the beta cells in response to insulin sensitivity are seen as increased cellular glucose metabolism, non-esterified fatty acid (NEFA) signaling, and sensitivity to incretins (Kahn, 2006). Incretins are hormones produced in the intestinal mucosa to enhance insulin response following oral administration of glucose.

Not only is an increase in insulin secretion seen, but there is also an increase in the volume of the beta cells. Glucose and NEFAs may promote an increase in beta cell mass. Another potentially important route of beta cell mass regulation is increased signaling by insulin and/or insulin-like growth factor 1 (IGF-1). Activation of the insulin/IGF-1 receptor leads to phosphorylation of IRS-2 and downstream signaling through pathways including PI(3)K/protein kinase-B (PKB/Akt) and Ras, eventually leading to increased beta cell proliferation and decreased beta cell apoptosis (Kahn, 2006).

A healthy adaptive response to insulin resistance changes both the function and mass of the beta cell, and is efficient enough to maintain normal glucose tolerance. In the case of beta cell dysfunction, the result is impaired glucose intolerance, impaired fasting glucose, and ultimately, type 2 diabetes.

Insulin signaling pathway (Akt signaling pathway)

Akt is a serine/threonine-specific protein kinase that is important in multiple processes such as cell growth, survival, proliferation, and metabolism. There have been three homologous

isoforms identified: Akt1, Akt2, and Akt3. Akt2 is particularly relevant for diabetes research, as it is (Mackenzie, 2014) involved in cell-substrate metabolism. It is most abundant in insulin-sensitive tissues, and plays a major role in mediating insulin action on metabolism. In one study, Akt2 knockout mice were found to develop marked insulin resistance and later, diabetes (Boucher, 2014). Another study with L-DKO mice (Liver Double IRS1 and IRS2 gene Knockout mice) prevented Akt phosphorylation and ultimately resulted in hyperglycemia, hyperinsulinemia, insulin resistance, and hypolipidemia (Guo, 2014).

Akt signaling also encourages insulin-stimulated glucose uptake in muscle and adipose tissue by its ability to translocate GLUTs to the cell membrane. At the same time, it inhibits glucose release from liver tissue.

Streptozotocin

Diabetes can be induced in the pig models using the drug streptozotocin. Streptozotocin inhibits insulin secretion, thus leading to a state of insulin-dependent diabetes mellitus. The drug is toxic to the pancreatic beta cells by alkylating them. The specificity of the drug is due to the selective cellular uptake and accumulation of streptozotocin in the pancreatic beta cells (Lenzen, 2008).

Streptozotocin is a nitrosourea, conferring upon its lipophilicity with reasonable tissue uptake through the plasma membrane. It is selected by GLUT2 glucose transporters found in plasma membranes. This makes other organs that contain this transporter besides the pancreas susceptible to damage by streptozotocin, particularly the kidneys and the liver (Lenzen, 2008).

The toxic activity of streptozotocin comes from the DNA alkylation activity and protein glycosylation activity of the drug (Lenzen, 2008). This leads to fragmentation of the DNA,

causing repair mechanisms to overactivate, depleting ATP stores, and ultimately causing necrosis of the beta cell.

Initially, the insulin biosynthesis, glucose-induced insulin secretion, and glucose metabolism are affected, with glucose transport being unaffected by the presence of the drug. Later on, as the effects of the drug manifest as reduced gene expression and protein production, both glucose transport and glucose metabolism are negatively affected.

Akkermensia muciniphilia

Gut microbiota such as *Akkermensia muciniphilia* have been known to interact with the body's glucose metabolism in a number of ways. In one way, the metabolites such as short-chain fatty acids produced by the microbiota-assisted fermentation of polysaccharides affect levels of several gut hormones involved with glucose and energy homeostasis. The metabolites circulate in the blood and are able to affect the insulin sensitivity of peripheral tissues (Cani, 2014).

The microbiota also confer an anti-inflammatory effect onto the body. Toll-like receptors (TLRs) recognize molecular patterns specific to bacteria and other microorganisms, also known as pathogen-associated molecular patterns (PAMPs). When these TLRs are stimulated, what follows is an inflammatory response, cytokine production, and recruitment of acute inflammatory cells. A common PAMP is lipopolysaccharide (LPS), found in the cell walls of Gram-negative bacteria, which is known to lead to inflammation and insulin resistance in cases of obesity of type 2 diabetes (Cani, 2014).

A growing area of study is at the interface between the gut microbiota and the intestinal mucus layer. Particularly, *A. muciniphilia*, mucin-degrading bacteria found in abundance in the mucin layer, has been shown to have a negative correlation with body weight and prone to

restore metabolic balance and insulin resistance. Despite these promising results, there is no consensus on the link between *A. muciniphilia* and type 2 diabetes.

Much focus is being given to the role gut microbiota play in the overall health of a person. Probiotics such as *Lactobacillus* and *Bifidobacterium* are commercially available to consumers in order to alter the makeup of the gut microbiota and positively affect metabolism, immunity, and nerve response (Naito, 2018). This study of *Akkermensia muciniphilia* may ultimately lead to the development of a commercial therapy for diabetes involving this particular probiotic in the form of a pill. However, the current focus of this study is more basic research to verify and confirm the mechanism by which *A. muciniphilia* confers an anti-diabetic effect onto its host. Work beyond this study may have a more application-oriented focus, such as optimal delivery method and ingestion concentration for a patient.

Aim

The aim of this study is to observe the effects of the probiotic *Akkermensia muciniphilia* and the insulin signaling pathways in pigs with streptozotocin-induced diabetes. The streptozotocin-induced diabetes is similar in pathophysiology to type 2 diabetes, making it an appropriate diabetes model to use. The use of pig models for the study adds a novel aspect to the study and allows for the translation of results for the animals to human models due to the similarities in physiologies between the two species.

Experimental Design

The study will follow a 2x2 factorial design in which two variables will be observed simultaneously: the presence of the probiotic *Akkermensia muciniphilia* and the state of

streptozotocin-induced diabetes. The following table shows the groups and their respective configuration of variables.

Figure 1. Experimental Design Groups

Treatment		Streptozotocin	
		-	+
Akkermensia muciniphilia	-	A (control)	D
	+	C	B

Tissue samples from one pig per group were used in this preliminary study.

The methodology of this study focuses on *A. muciniphilia* as a possible preventative treatment for diabetes. The probiotic is administered into the animal first, followed by the induction of diabetes weeks later.

The proposed hypothesis is that Group C will exhibit the greatest activity of Akt phosphorylation among the four groups. Akt is a key marker in the insulin signaling response, and increased phosphorylation will point towards the therapeutic mechanism of *Akkermensia muciniphilia* against diabetes.

Materials and Methods

Reagents

Anti-phospho-Akt-S473, anti-phospho-Akt-T308, and anti-Akt antibodies were from Cell Signaling Technology (Beverly, MA).

Animal Models

A total of 20 male weanling pigs (Yorkshire x Landrace/Duroc) were purchased from a local commercial farm (Prestage Farms, West Point, MS) and randomly allotted into 2 groups housed in 20 feeding pens individually. Streptozotocin was injected intravenously into half of the 6-week old pigs for a single, one-time injection. The control group received injections of 25mL disodium citrate buffer. The experimental group received a 25mL injection of streptozotocin dissolved in disodium citrate buffer. 5 pigs per group with the same diet were randomly assigned to the *A. muciniphilia* or the heat-killed inoculation group. *A. muciniphilia* was added to the feed freshly and the pigs received the probiotics through the top-load method once every morning at an amount of 10^{11} cfu for another 4 weeks. Pigs were given free access to feed and water and checked twice daily. The experimental house had a controlled 12-hour light/dark cycle. Over the 6-week course, weekly changes in individual pig body weights and daily pen feed consumption were recorded. The pigs were then slaughtered at the on-campus abattoir and tissue samples of the pigs' liver, pancreas, and muscle were collected, frozen in liquid nitrogen, and stored at −80°C until analysis.

***A. muciniphilia* preparation and inoculation**

A. muciniphilia MucT (BAA-835, biosafety level 1) from ATCC was grown anaerobically in a mucin-based basal soft agar medium as described by ATCC's recommended protocol. Half of the pigs were fed with *A. muciniphilia* by a top-load approach directly adding the bacteria to the pig's meal in the morning. Pigs were fasted for 1 hour and then fed with 10g of feed containing freshly added *A. muciniphilia*. Half of the pigs were given a daily does of 10^{11} cfu suspended in 1mL sterile anaerobic PBS for 4 weeks, and the other half of the pigs were given the same does of heat-killed *A. muciniphilia*. The cultures were maintained under strict

anaerobic conditions. Successful administration of *A. muciniphilia* was confirmed by qPCR analysis of fecal *A. muciniphilia* before and after inoculation between control and treatment groups.

Preparation of Protein Extracts and Western Immunoblots

Approximately 0.250g of tissue was ground using a grinder. Samples were kept cool with liquid nitrogen. The ground tissue samples were then mixed into solutions containing 970 µL RIPA lysis buffer, 10 µL PMSF solution, 10 µL sodium orthovanadate solution, and 10 µL protease inhibitor cocktail solution. The samples were homogenized thoroughly and kept on ice for 30 min, with occasional vortexing throughout the 30 min. After centrifugation at 16,000 x *g* for 30 min, soluble protein concentration was determined by using the BCA assay (Pierce). After normalizing the crude homogenate amounts for 50 µg/10 µL of protein concentration, the homogenate was resolved on 10% Tris/glycine SDS polyacrylamide gels at 60 V for 30 min, then 100 V for 90 min. The gels were then transferred to nitrocellulose, probed at 4°C with rabbit polyclonal anti-phospho-Akt-S473 antibody (1:1,000), rabbit polyclonal anti-phospho-Akt-T308 antibody (1:1,000), or rabbit polyclonal anti-Akt antibody (1:1,000). Results were quantified by densitometry, and the ratio of phospho to total proteins was determined in arbitrary units and expressed relative to the ratio for sham-treated animals prepared and blotted at the same time.

Data/Results

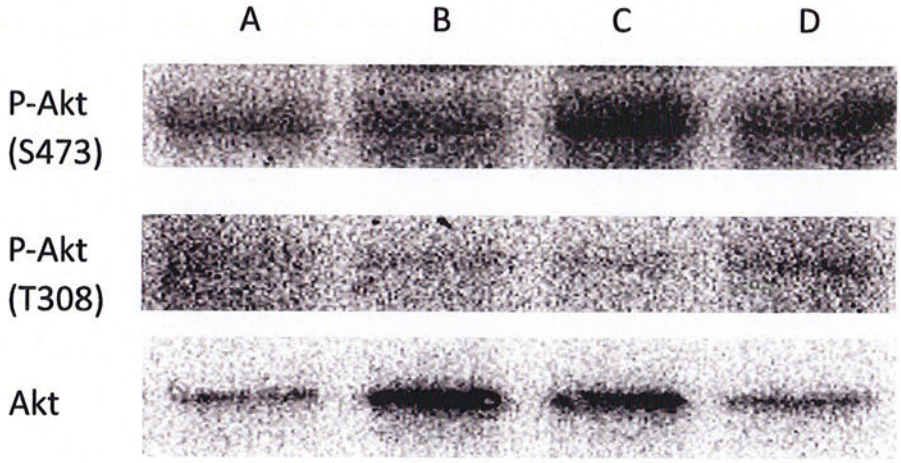

Figure 2. Representative Western blots of muscle tissue samples

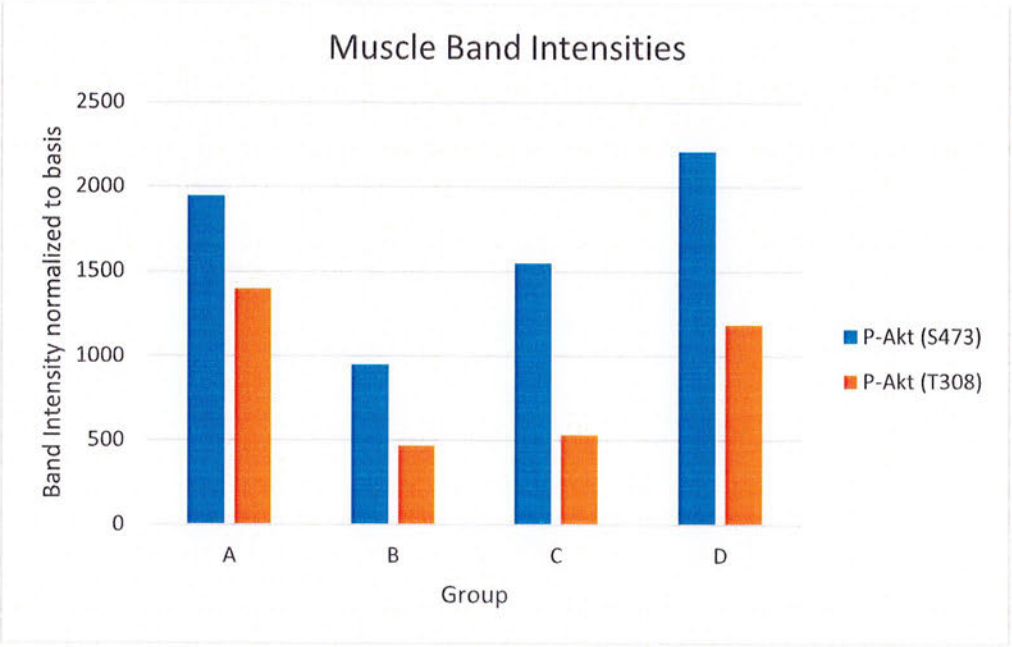

Figure 3. Phosphorylation of select insulin signaling intermediates in muscle tissue samples

For all experimental groups in among the muscle tissue samples, Akt phosphorylation activity was lower for all phosphorylation sites than activity found in the double negative control group A except for phosphorylation of S473 in the probiotic-negative, diabetic-positive group D. The diabetic group shows increased phosphorylation activity of S473 by a small amount. The

probiotic-positive, diabetic-negative group C shows less phosphorylation activity than group D. The double positive group B shows the least phosphorylation activity among the groups tested.

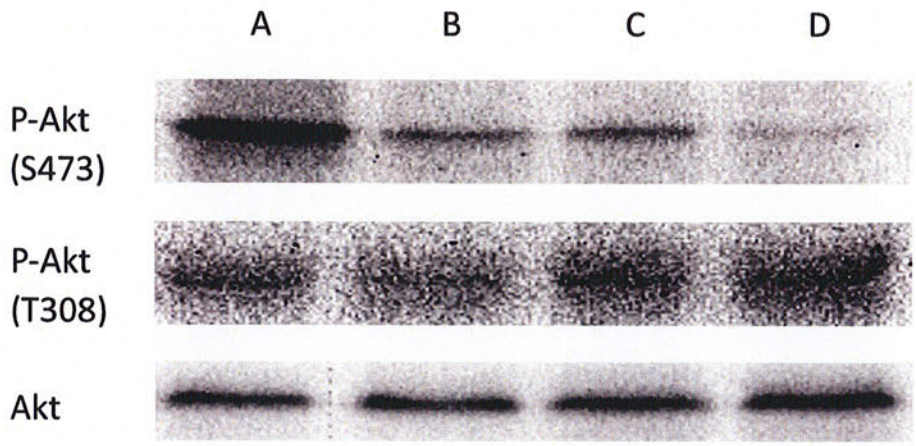

Figure 4. Representative Western blots of pancreas tissue samples

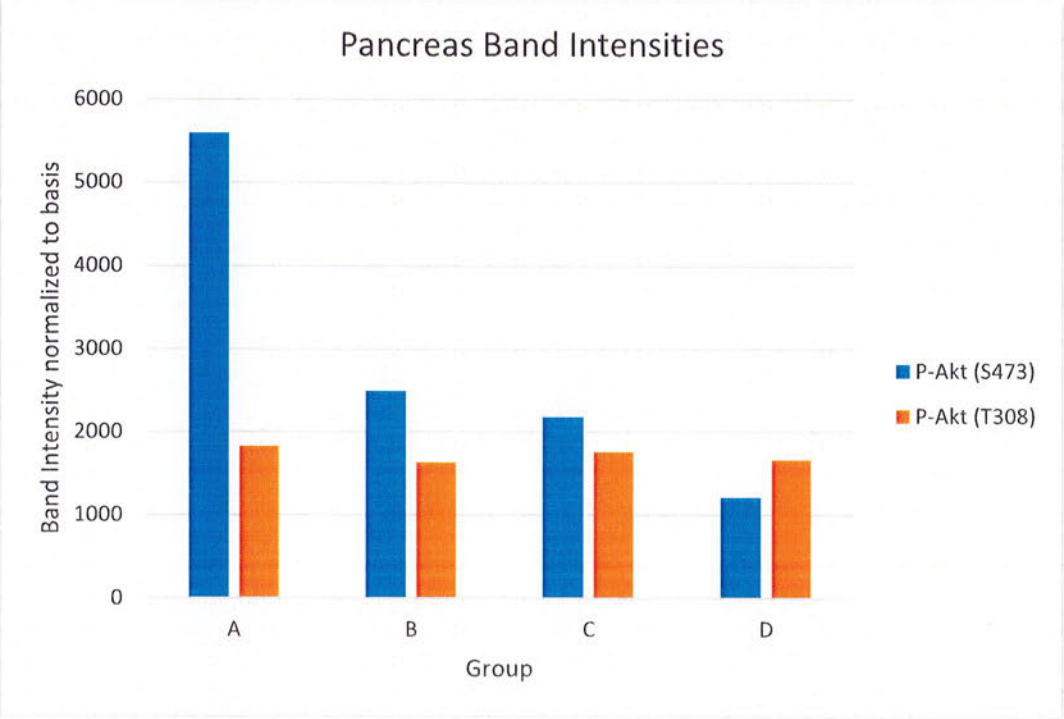

Figure 5. Phosphorylation of select insulin signaling intermediates in pancreas tissue samples

From the data for the pancreas tissue samples, there is no marked difference between the experimental groups in the phosphorylation of Akt at the T308 site. The probiotic-positive group C is close to the control group A in terms of Akt T308 phosphorylation. The diabetic groups B

and D present slightly less phosphorylation for this site. For the S473 site, the phosphorylation levels are lower in the diabetic group D than in the probiotic group C. Group B shows higher phosphorylation activity at the S473 site than in group C. The control group phosphorylation at S473 is drastically higher than the rest of the groups.

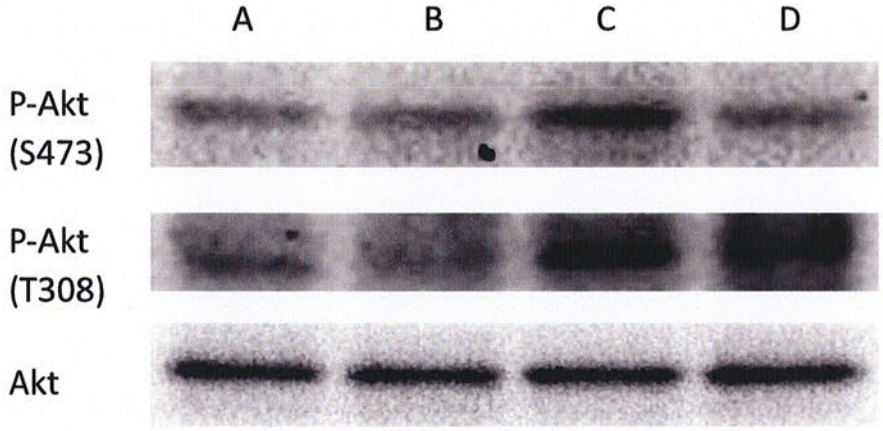

Figure 6. Representative Western blots of liver tissue samples

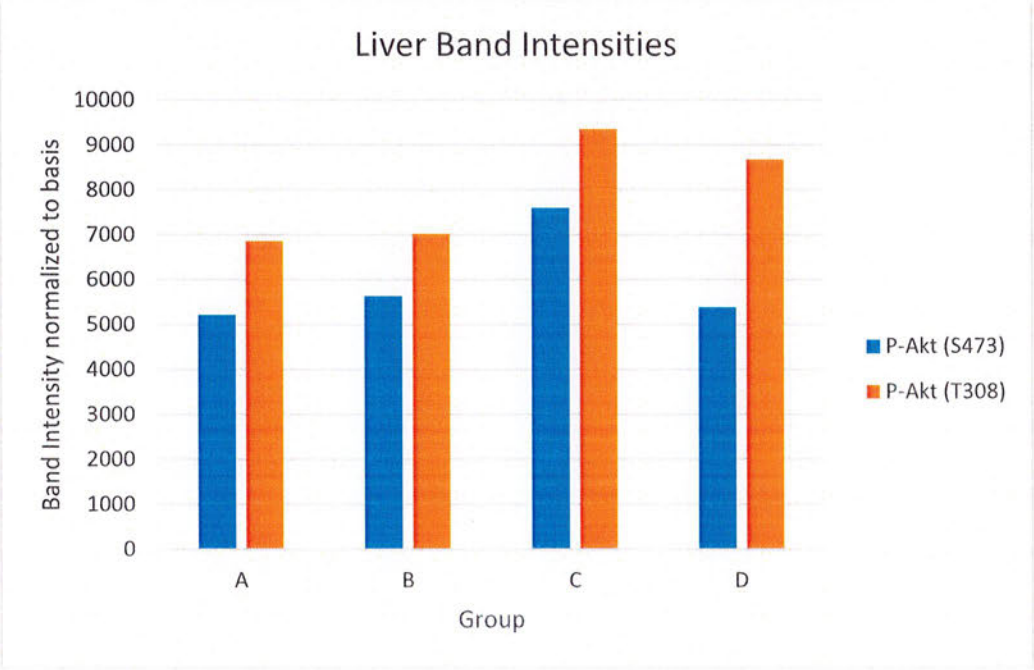

Figure 7. Phosphorylation of select insulin signaling intermediates in liver tissue samples

From the liver tissue data, the probiotic group C shows the highest levels of Akt phosphorylation for both sites relative to the other three groups. The double positive group B shows slight increase in phosphorylation activity for both sites relative to the control group. The diabetic group shows a slight increase in phosphorylation activity in the S473 site and a marked increase at the T308 site. Overall phosphorylation for the diabetic group was still lower than the probiotic group.

Discussion

The Akt signaling pathway is important in regulating the glucose metabolism of a cell. In pancreatic beta cells, as Akt phosphorylation is increased, beta cell proliferation is increased and apoptosis is decreased. In tissues that utilize large amounts of glucose, such as muscle tissue or liver tissue, the Akt signaling pathway regulates glucose metabolism.

Akkermensia muciniphilia has been found to show an anti-diabetic response through its anti-inflammatory behavior. Its proximity to the mucin layer of the intestine gives it access to the circulatory system, allowing it to better circulate its metabolites to peripheral targets and increase its anti-diabetic influence beyond the local area of the intestines.

In a study done in human models of diabetes, phosphorylation activity of Akt at the S473 and T308 sites were shown to be less in the diabetic groups than in the lean control groups (Tonks, 2013). This differs with the data in this study in that the induced diabetic group showed higher S473 phosphorylation and lower T308 phosphorylation than with the control group. The nature of the results from the human muscle study may be the result of the tissue collection method, as a muscle tissue biopsy was performed 30 min and 145 min after a glucose solution intravenous injection into the subject. The imprecise timing of the harvesting of the tissue from

the pig models in this study may have affected the representation of the phosphorylation activity found in the muscle tissue.

Another area of difference to note between the Tonks study and this study is the model of diabetes used. Streptozotocin results in pancreatic beta cell death, which closer mimics the pathophysiology of type 1 diabetes or late stage type 2 diabetes than it does early stage type 2 diabetes. The patients of the Tonks study had type 2 diabetes, which presumably did not entail complete pancreatic failure but rather insufficient pancreatic action in regards to insulin secretion.

As for the effect of the presence of *A. muciniphilia* on the phosphorylation of Akt in muscle tissue, a study done in mice models suggests that its presence increases the phosphorylation activity of Akt at the S473 position in both liver and muscle tissue (Zhao, 2017). This contradicts the findings in this study, as the probiotic group shows less Akt phosphorylation activity than in the control group. The size of the animal models may have played a role in this, as the anti-inflammatory effects of the probiotic may have not within the gut longer enough to spread systemically.

The pancreas data is shows much less variation than the muscle data. As expected, the probiotic group showed increased Akt phosphorylation activity, albeit slight, than the diabetic group at both the S473 and T308 sites. As the pancreas is the location for beta cells that produce insulin, it is closely involved with glucose metabolism, which Akt plays a large role in regulating. The induction of the diabetic state via streptozotocin would act opposite to the cell growth promoting tendencies of the Akt signaling pathway, which is why the phosphorylation activity in the diabetic group is lower than the control group. The extremely high phosphorylation at the S473 for the control group may be due to the insulin-secreting purpose of

the pancreatic tissue, or moreso may be a result of differing times of slaughter after feeding similar to the muscle band results.

The liver plays a major role in lipid synthesis and insulin sensitivity. As such, the data show increased phosphorylation activity in both the S473 and T308 sites for the probiotic group, concordant with the earlier study showing increased phosphorylation activity due to the introduction of *A. muciniphilia* to the animal (Zhao, 2017). The diabetic group is lower in phosphorylation than the probiotic group, but at the same time higher than the control group. This may be a result of mistimed slaughtering of the animals in between feedings or possibly an inadequate dose of streptozotocin for the pig.

As Akt is involved in cell metabolism, the treatments may have had an effect on Akt levels in the different tissues, making Akt possibly an unreliable marker to use as a basis.

There is much more work to be done to complete this study of the relationship between *Akkermensia muciniphilia* and diabetes. With the samples and data used for this study there are improvements to be made. More trials could be done with more pigs to confirm the effect of the variables on different specimens as well as to gain a better idea of the statistical significance of the results. The protein images could be cleaned of background signal to allow for more accurate quantification and comparisons of the data. A final area of improvement within the confines of this particular study involves monitoring the amounts of the basis Akt in response to the different experimental treatments. Future studies can focus on utilizing other common markers uninvolved in cell metabolism such as beta-actin.

More Western blotting along the insulin signaling pathway can be done in order to be certain of the effects of *A. muciniphilia*. Markers of note include Akt substrate of 160kDa,

glycogen synthase, and insulin receptor substrate. The study could also be replicated with another method of diabetes induction so as to avoid the shortcomings of streptozotocin, such as a diet-induced obesity which may more closely mimic the pathophysiology of type 2 diabetes. Tissue biopsy could also be done following glucose injection to measure insulin sensitivity following a defined time. Additionally, the tests performed in the study can be complemented with data of blood glucose levels and blood plasma biochemical testing for a more context for the results. Finally, the therapeutic effects of the probiotic may be tested by administering the probiotic after the induction of diabetes in a specimen.

Conclusion

In conclusion, this study highlights the need for more work to be done on the subject. In this preliminary study, mixed or inconclusive data were obtained relative to other studies. By observing the Akt phosphorylation activity in the insulin-sensitive tissues of the pancreas, liver, and muscle, there is some link between the presence of *Akkermensia muciniphilia* in the gut microbiota and the Akt signaling pathway as a part of the greater insulin signaling pathway.

The work done in this preliminary study, despite providing a mixed bag of results, points us closer towards a possible preventative measure against type 2 diabetes, especially among the overweight and obese. Much prior work has been done in confirming the positive effects of the anti-inflammatory, anti-diabetic probiotic *Akkermensia mucinphilia* in small animal models such as mice, but few have brought examined the effects of the probiotic on animals with much more physiological relevance to humans such as pigs. The use of a pig model in this preliminary study allowed us to better predict the effects of the probiotic on a human model, as the physiologies of the two species are much more similar than between mice and humans. The sequence of

variables changed in the animal allowed us to investigate the use of the probiotic as a preventative measure, as the probiotic was administered before the induction of diabetes in the animal. Ultimately, with additional research, *A. muciniphilia* may be commercially available as a probiotic supplement to combat the onset of diabetes.

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