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**Observing the Effects of *Aspergillus oryzae* on Combating the Consequences of Heat Stress
in Lactating Dairy Cows**

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Abstract

With profit margins becoming ever slim in the dairy industry, searching for new methods to minimize the negative effects of heat stress on lactation and milk yields is important. The aim of this study is to provide dairy cows with a prebiotic fermentation extract of *Aspergillus oryzae* to assess its potential to serve as a solution in this problem. To observe the effects of this prebiotic, a nutritive supplement containing fermentation extract of *A. oryzae* was fed to lactating Holstein cows in four different concentration groups – 0 g/day, 3 g/day, 6 g/day, and 18 g/day – under heat stress conditions. Response of the cows to this prebiotic in association with heat stress was quantified using the following data points: milk and blood samples; body weights and body condition scores; respiration rates; and rectal, udder, and vaginal temperatures. It is predicted that as a result of the prebiotic supplement, milk yield and milk protein will increase. It is also predicted that as a result of the supplement treatment, there will be decreased levels of acute phase protein synthesis from the liver as well as decreased cytokines present in blood samples.

Due to the confidentiality agreement between researchers and supplement company, actual results cannot be discussed within this thesis. However, possible results and their interpretations will be outlined and examined. Reasoning behind the predicted results as well as known physiological reactions to heat stress and prebiotic supplements will be used in an effort to justify those predicted results. Prior to the beginning of the study, involved personnel underwent a series of training programs in order to properly comply with the University of Tennessee ethics code. Members were enrolled in the Occupational Health Program (OHP), underwent emergency preparedness training, and participated in Institutional Animal Care and Use Committee (IACUC) training.

Introduction

Heat stress is defined as a series of negative physiological effects inflicted on the individual as a direct result of increased environmental heat and/or humidity levels.

Environmental temperatures of 80°F and higher and humidity levels above 20% can lead to an increase of the internal temperature of the individual which can further negatively affect the individual's metabolic functions and overall health (Keown, et al., 2019). Some of the first signs of heat stress include increased respiration rate and decreased eating. In dairy cows, some of the most prominent effects are seen in a decrease in milk production and a decrease in conception rates.

Physiological Effects of Heat Stress on Lactation in Dairy Cows

The rectal temperature of a healthy, non-heat stressed cow is expected to fall between approximately 100.6°F and 102.4°F with an average of 101.5°F (Wenz, et al., 2011). Heat stress in cows begins approximately when rectal temperature reaches 102.7°F, although this varies depending on what internal temperature is normal for the individual cow (Srikandakumar, et al., 2004). When heat stress beginnings to occur, blood flow to the placenta is reduced as blood flow is directed towards the extremities in an effort to cool the body core and internal organs (Sawka, et al., 1993). As lactation is largely dependent on proper functioning of the placenta, decreased blood flow to the placenta can lead to decreased levels of lactation and mammary gland efficiency (Hansen, et al., 2007). In addition, decrease in feed intake due to heat stress negatively affects lactation as lactation is a metabolically costly activity, requiring the animal to eat for proper feed conversion. Lactation is a metabolically costly process not essential for the survival of the individual and is often one of the first processes to be halted when the body is placed under stress or when feed intake is decreased.

Heat stress alone is estimated to be able to cause a milk production decrease of up to 10% in a dairy cow (Hoard's Dairyman, et al., 2013). This production decrease can greatly be attributed to decreased appetite as a result of heat stress. With less feed intake, less energy is available to the animal and, thus, less milk is produced. With this in mind, in 2003, heat stress was estimated to result in an annual average loss of nearly \$900 million in just the dairy industry (St-Pierre, et al., 2003). Considering inflation and the current state of the dairy market, this pressing issue warrants serious research to find better solutions for counteracting the effects of heat stress.

***Aspergillus oryzae* and Its Potential Benefits**

In an effort to correct the effects of heat stress on eating habits and, thus, increase milk production by increasing feed intake, prebiotics are a logical choice to supplement into the cow's diet. The addition of a prebiotic may act as a direct way to increase nutrients and energy available to the animal. A prebiotic is defined as a nondigestible dietary additive which benefits the gastrointestinal microbiota by supporting the growth of advantageous microbial species (Hutkins, et al., 2016). Because gut microbes are an absolutely essential part of rumen digestion, it is logical to infer that by fortifying this microbial community, digestion will be made more efficient, leading to an increase in metabolic efficiency and, thus, lactation.

Aspergillus oryzae is a fungus used commonly in Japanese cuisine; also known colloquially as "koji", this mold is often added to dishes as fermentation processes can give a dish the signature and sought-after umami flavor (Kamin, et al., 2017). However, in the animal world, *A. oryzae* is starting to be used as a prebiotic supplement and is being marketed particularly in equine nutrition as an appetite stimulant. While there exists one particular *A. oryzae* product aimed at increasing feed digestibility in dairy cows, resources are limited on how

effective the addition of this fungus is in supporting milk production within dairy cows. Therefore, the purpose of this study was to assess the effectiveness of a *A. oryzae* prebiotic supplement in combating the effects of heat stress on milk production within a dairy cow herd.

Materials and Methods

Project Set-Up

A total of 48 lactating Holstein cows were used for the purposes of this research project. They were separated into two pens, both on the farthest Northwest side of the East Tennessee Research and Education Center (ETREC) – Little River Dairy barn located in Walland, TN. These pens consist of a free stall system with sand bedding and both contained water misters and a fan system. These heat abatement tools were connected to a hobo data logger which analyzed temperature and humidity levels within the barn. The heat abatement system was allowed to function normally during the first period of the study – which lasted for 10 days. However, during the second period of the study – also called the heat stress challenge, which lasted for 25 days – these heat abatement tools were deactivated from 9am to 9pm, unless temperatures rose above 93°F, and activate from 9pm to 9am, unless temperatures dropped below 85°F. The barn doors were also able to be closed for days when a lower ambient temperature necessitated their closure to maintain a heat stress environment. Cow health was monitored throughout the experiment period. If a cow were to show signs of extreme heat stress outside of the acceptable level of this experiment, she would be moved to cooling pen until body temperature returned to an acceptable level, at which point she would be returned to the experiment.

Cows were separated into 4 different groups of 12 cows each, keeping in mind an even mixture of pregnancy status, parity – number of previous pregnancies –, days in milk – the number of days since the start of lactation –, and average milk yield across the groups.

Treatment Explanation and Diet

Prior to the heat stress challenge, cows underwent a training period in order to learn how to use the Calan feeding gates. Calan gates are designed to feed individual diets within a group housing environment. Each cow wears a “key” around her neck which electronically unlocks only her assigned gate, ensuring that only she can eat out of her individual feed tub. At the start of training, cows were not assigned keys and the Calan gates were not locked but allowed to open freely. The purpose of this step was to allow the cows to become accustomed to pushing the gate open and to placing their head into the small space to eat – an act that is not natural to them. Once all cows had become accustomed to this system, they were assigned individual gates, and key collars were fastened around each cow’s neck. Next, the cows underwent another training period in order to learn which gate their key would open and to become accustomed to eating out of this particular feed tub.

During this initial, pre-heat stress challenge training period, the cows were fed a total mixed ration (TMR) consisting of hay, grain, corn silage, and rye silage in order to acclimate to their new diet. The TMR was used throughout the study and was fed at approximately 6am and 4pm each day. Prior to the heat stress challenge, baseline values for feed intake were recorded to calculate the amount to be fed to each individual cow throughout the experiment; baseline values for milk production were also determined for comparison to heat stress values collected later in the study. Milking took place twice a day before each feeding session. Feed was dispensed using a Calan Data Ranger which is a feeding system which dispensed feed by weight in pounds.

Prior to each morning feeding, “weigh-backs” were taken which is a process where feed left over from the previous day was collected from each individual feeding tub. This refusal feed was weighed using the Data Ranger and was recorded.

Once training was completed, the heat stress challenge, or experimental period, began and lasted for 25 days. During this period, heat abatement systems were turned on and off as previously described and the barn doors were lowered when necessary in efforts to maintain a reasonable heat stress environment within the test groups. The *A. oryzae* prebiotic supplement was introduced to the study based on the four different groups. The first group served as a control and did not receive any prebiotic supplement. The second group received 3g each day, the third 6g each day, and the fourth 18g each day. These supplements were divided evenly between the morning and evening feedings and were introduced to the TMR using a Top-Dressing method. Top-dressing means that once the appropriate amount of TMR was weighed out into the tub, the individual supplement was sprinkled on the top and mixed within the top layer of TMR. Supplements were weighed out prior to each feeding and placed into individually Ziploc bags labeled with the gate number of the cow to which it should be fed.

Data Collection

Periodically throughout the experiment, milk samples were collected as were feed samples of each dietary ingredient, TMR, and refusal TMR. Throughout the experiment, heat stress was quantified using rectal temperatures – using a rectal probe thermometer –, udder temperatures – using a thermal imaging gun –, vaginal temperatures – using CIDRs loaded with temperature loggers –, and respiration rates – using visual counts. Data for body weight was also collected for each individual cow at the beginning and end of the study.

The last six days of the heat stress challenge were the primary data collections days. On the first data collection day, milk samples were collected in the morning and evening within the milking parlor; respiration rates and body temperatures were also recorded in the morning and evening. On day two of data collection, feed samples were collected and, again, milk samples as well as respiration rates and body temperatures were collected in the morning and evening. Day three of data collection consisted of collecting milk samples, respiration rates, and body temperatures in the morning and evening. The fourth day of data collection consisted of collecting milk samples, respiration rates, and body temperatures in the morning and evening; blood samples were also collected in the morning. The blood collection procedure consisted of disinfecting the collection site using an alcohol swab, venipuncture using the coccygeal – or tail – vein with a 16g needle and hub, and collection using a green top tube. The tube was removed, then the needle, and pressure was applied to the collection site to ensure the site did not continue to bleed. The sample was then inverted several times to ensure mixing of the sample and heparin and was stored on ice.

On the fifth day of data collection, feed samples were collected; milk samples, respiration rate, and body temperatures were collected in the morning and evening; blood was collected in the morning, and body weights and body condition scores were collected in the morning. Body condition score was assessed on a 5-point scale with a score of 1 indicating an extremely thin cow and a score of 5 indicating a severely obese cow. Healthy dairy cows at their peak lactation typically receive scores between 2.5 and 3.5. On the final data collection day, blood samples were collected in the morning for use in the Ex Vivo challenge, which will be discussed in the following section.

Data Analysis Methods and Ex Vivo Challenge

Milk samples were analyzed at the University of Tennessee – Dairy Herd Improvement Association (DHIA) for the main purpose of collecting data on lactose, fat, protein, non-fat solid components – which consisted of protein and lactose –, and somatic cell counts within the milk. Somatic cell counts are a reflection of present immune responses as they refer to the number of leukocytes – or white blood cells – that are present within the sample. Milk protein and fat; lactose; and acetate, butyrate, and propionate – which are volatile fatty acids produced by rumen microbes – were analyzed.

Plasma samples were analyzed first to look at non-esterified fatty acids – also called free fatty acids – which is also a measure of all fatty acids within the blood. “Non-esterified” refers to the fact that these fatty acids are not connected to a glycerol backbone as is the case with esterified fatty acids. Plasma samples were also analyzed for urea-nitrogen levels which act as a marker for breakdown of muscle. In addition, dietary extract was analyzed and related to rectal temperature and respiration rates. Finally, plasma was analyzed for Acute Phase Proteins which are pro-inflammatory markers, meaning they are released prior to an inflammation response.

For the Ex Vivo challenge, whole blood was used to generate the most natural and whole response possible. All samples were treated with exactly the same amount of lipopolysaccharide (LPS) – an endotoxin which is released under stress conditions. The stimulation of the whole blood samples with LPS in an ex vivo environment allowed for standardized stress conditions across all samples tested. The addition of this exotoxin stimulated increased leukocyte release within the blood and, thus, increased cytokine production and release within the blood sample. These biological products were measured for each treatment in an effort to not only measure

immune response to LPS but also to determine if the population of leukocytes – neutrophils, monocytes, lymphocytes, basophiles, and/or eosinophils – would vary between treatment groups

Finally, isolated RNA from the Ex Vivo challenge was subjected to PCR with a focus on the particular cytokines present in the samples. Cytokines associated with inflammation – particularly IL-1 β , IL-6, and TNF- α – were examined as they are proinflammatory agents.

Results

Confidentiality Disclosure

Due to the confidentiality agreement drafted between researchers and the company which supplied the *A. oryzae* prebiotic supplement used in this study, actual results of this study are not yet privy to the public or to publication within this thesis. However, for the purpose of conclusion and expanded understanding of this topic, expected results as well as other potential results will be discussed.

Expected Results

The treatment of fiber extract from *A. oryzae* fermentation is aimed at increasing feed digestibility through its prebiotic nature and, thus, increasing nutrient availability to the animal. Protein levels within the milk are predicted to increase as the amount of supplement fed increases (**Figure 1**). Milk lactose will also increase, and, thus, milking yield will increase with increased supplement fed (**Figure 2**).

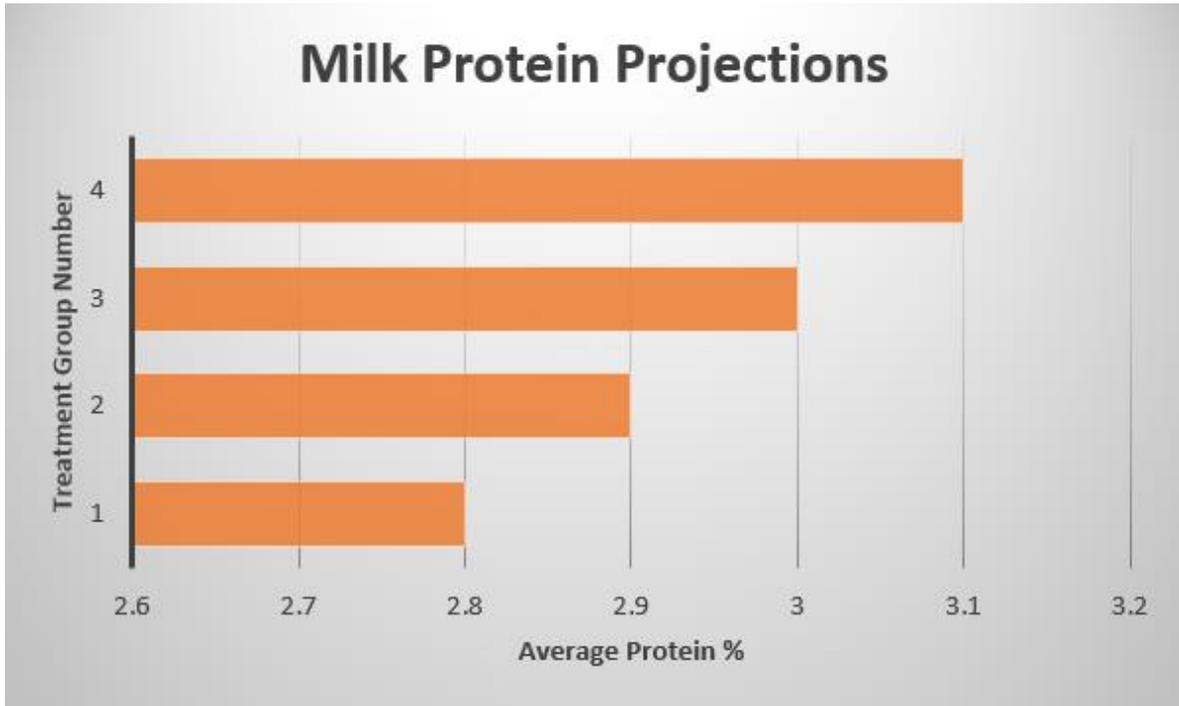


Figure 1. Predicted Average Milk Protein Percent Across Treatments*

Treatment Group Number is detailed on the y-axis where these values are defined as 0 g/day supplement fed in Group 1, 3 g/day in Group 2, 6 g/day in Group 3, and 18 g/day in Group 4. Milk protein percent is projected to increase as the amount of supplement fed per day increases from group to group.

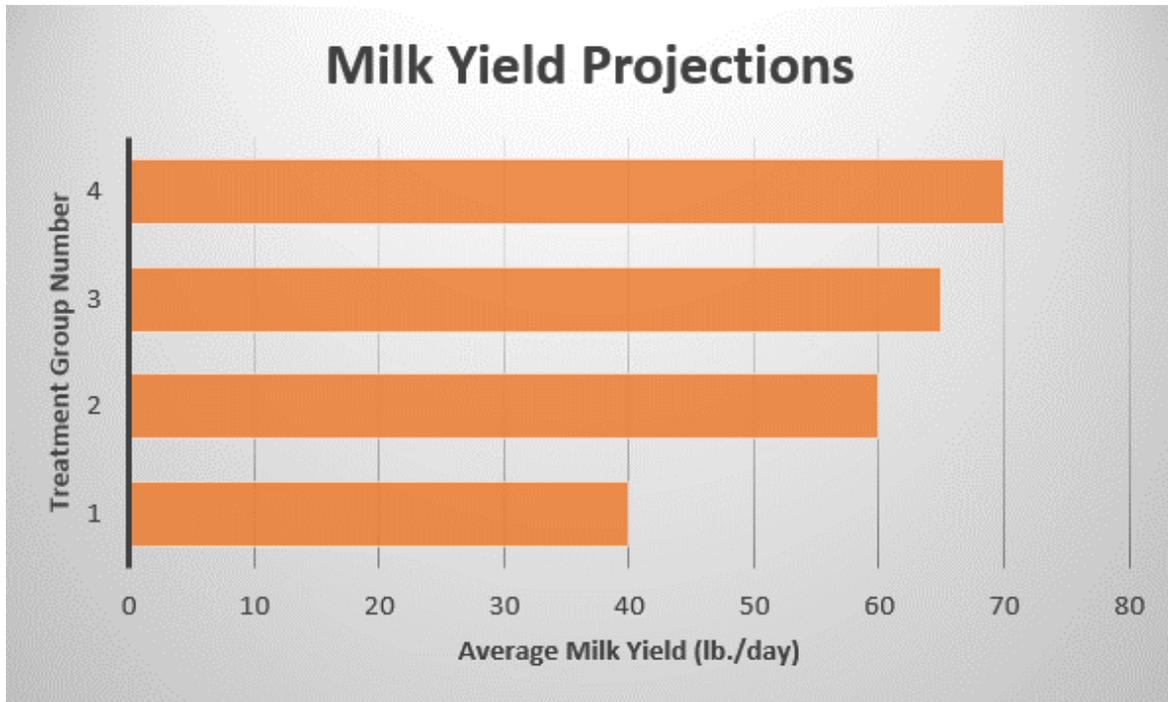


Figure 2. Predicted Average Milk Yield Across Treatments*

Treatment Group Number is detailed on the y-axis where these values are defined as 0 g/day supplement fed in Group 1, 3 g/day in Group 2, 6 g/day in Group 3, and 18 g/day in Group 4. Milk yield is predicted to increase as the amount of supplement fed increase across groups.

As for plasma sample analysis results, although difficult to predict how this particular treatment will affect plasma values, there exists the potential for a decrease in urea-nitrogen with increased supplement provided. There is no predicted change in the use of fatty acids. With increased supplemental treatment, acute phase protein synthesis is predicted to decrease from the liver. Finally, with the Ex Vivo challenge, those individuals that received no supplement – Group 1 – are predicted to have more monocytes and lymphocytes resulting from LPS stimulation while those samples from individuals that received supplement will show a decrease in monocytes – pre-macrophages – and lymphocytes and, thus, a decrease in cytokines and, thus, again, a decrease in acute phase proteins.

Other Potential Results

Alternative results for this experiment function to suggest that the prebiotic supplement did not work as intended for what ever reason. If this occurred, there would not necessarily be an increase in average milk protein percent (**Figure 3**), and there would not necessarily be an increase in acetate and/or butyrate levels. There would not necessarily be more energy available to the animal, so milk lactose would not increase as a result, and milk yield would not necessarily be increased as a result of increasing amount of supplement fed (**Figure 4**).

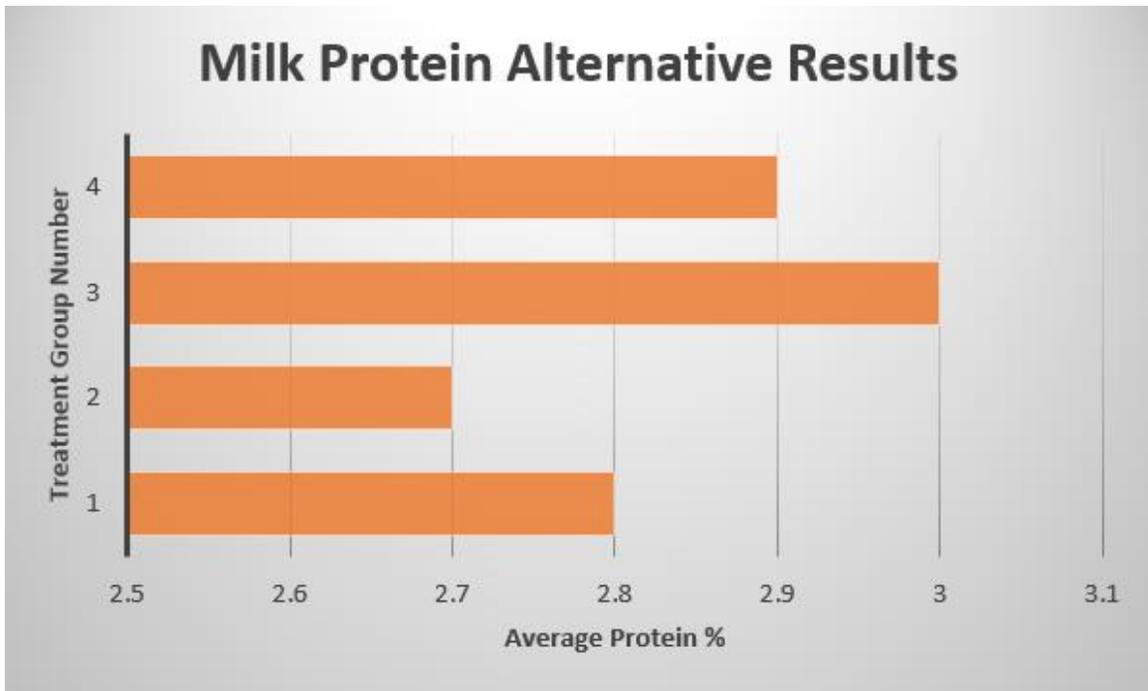


Figure 3. Milk Protein Alternative Results*

Treatment Group Number is detailed on the y-axis were these values are defined as 0 g/day supplement fed in Group 1, 3 g/day in Group 2, 6 g/day in Group 3, and 18 g/day in Group 4. The values along the x-axis are random and function to suggest the possibility that there is no correlation between increased feeding of the supplement and increased average milk protein percent.

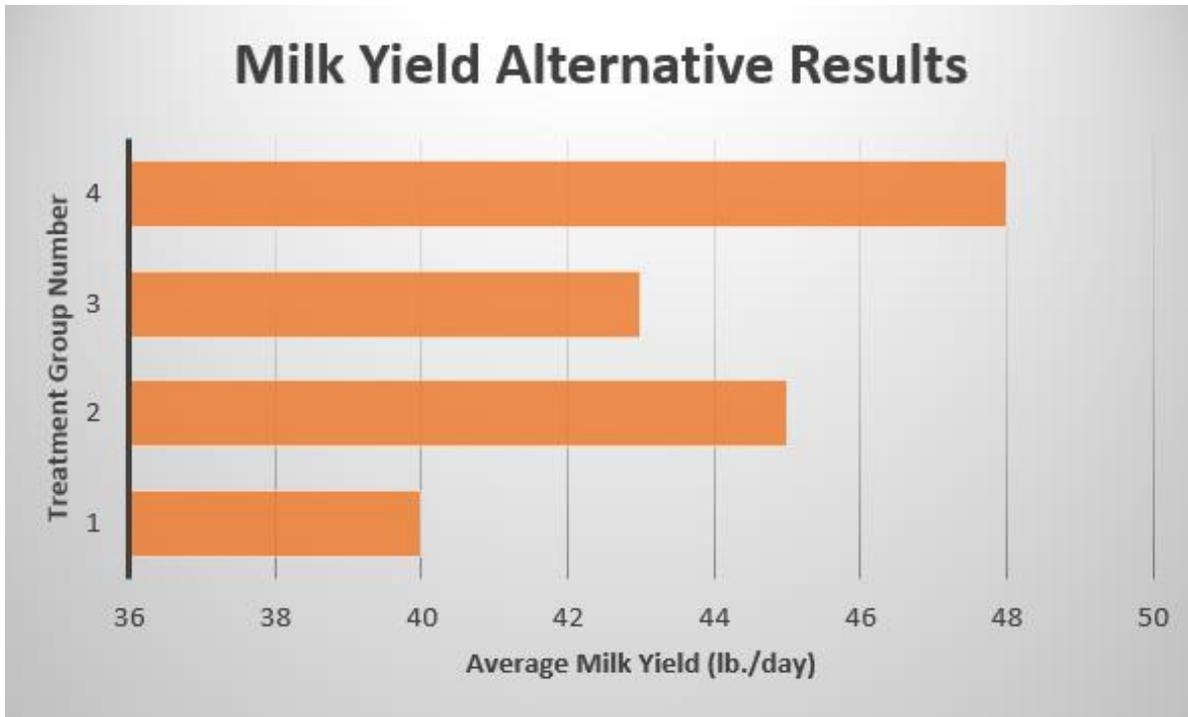


Figure 4. Milk Yield Alternative Results*

Treatment Group Number is detailed on the y-axis where these values are defined as 0 g/day supplement fed in Group 1, 3 g/day in Group 2, 6 g/day in Group 3, and 18 g/day in Group 4. The values along the x-axis are random and function to suggest the possibility that there is no change in milk parameters as a result of the supplement.

***These are hypothetical results for demonstration purposes only. These do not represent actual results.**

Similar to milk results, alternative plasma contraction results would suggest that there is not a correlation between the prebiotic supplement and fatty acid levels, acute phase proteins, monocytes and lymphocytes present, and, thus, cytokine production within the blood samples across different treatment groups.

Discussion

Interpretation of Various Potential Results

An increase in feed digestibility due to the prebiotic would lead to an increase in nutrient absorption. With increased nutrients – and, thus, increased amino acids – supplied to the animal, we would expect to see a resulting increase in milk protein. Acetate is a major contributor to milk fat synthesis while butyrate is important in immune function. An increase in milk fat levels could be due to an increase in acetate and butyrate level because *A. oryzae* is particularly beneficial to fibrous bacteria. Again, with increased energy available to the animal, milk lactose increases, increasing water drive into the mammary gland, finally increasing milk yield.

Due to the fact that esterified fatty acids are always converted to non-esterified fatty acids when they are released from adipose tissue into the blood stream, non-esterified fatty acids are only present in blood. The process is common when an individual undergoes stress. However, in the case of heat stressed animals, fatty acids are actually not used for energy requirements. These individuals break down muscle instead in the effort to reduce internal temperature, and they actually store more fat as it generates less heat than muscle. The negative effect of heat stress – breaking down muscle – may be limited, and, thus, metabolism will be shifted to fatty acids instead when digestibility is improved with the addition of the supplement. If muscle breakdown is limited, we would expect to see a potential decrease in urea-nitrogen – which, again, is a marker for this muscle breakdown. A decrease in urea-nitrogen would also suggest that more amino acids are begin utilized in metabolism and absorbed as a result of dietary protein.

Heat stress often induces an immune response to the environmental conditions; this is a challenge as increased immune function is beneficial but the increased inflammation that often accompanies it is not. Inflammation can be damaging to tissues and it requires glucose which takes away from glucose that could otherwise be utilized in milk production. The prebiotic supplement is predicted that the prebiotic supplement will decrease the number of leukocytes originally formed, thus decreasing cytokines produced and further decreasing acute phase protein synthesis from the liver, leading to lower levels of inflammation. In short, decreasing the inflammatory response could lead to more available glucose and, thus, higher milk production. Alternative results that contradict predicted results would function to suggest that the supplement does not affect the cow as intended or that there are other, unexpected factors acting on the efficacy of the supplement.

Significance of this Project and Potential Benefit to the Agricultural Industry

Milk protein and fat are important markers of milk quality and are indicative of optimal metabolism within the animal. Lactose is especially important to analyze as this component is the driving factor in recruiting water into the mammary gland, thus, increasing milk yield. In other words, higher levels of lactose are associated with higher milk production. This supplement has the potential to reduce milk production loss due to heat stress through increasing the energy available to the animal, promoting metabolism of fatty acids over muscle, and decreasing the inflammatory response to the environment. Aside from the obvious benefit to the health and welfare of dairy cows that this product presents, it is also highly beneficial to the industry as a whole. Profit margins in the dairy industry are currently slim, and many producers are struggling to breakeven. It is important to reduce milk production loss in order to maximize production efficiency and profit.

Further Questions

Further studies into this prebiotic supplement in other species such as sheep and goats in order to assess its efficacy in combating the effects of heat stress on their lactation would be potentially beneficial. Examining the effect of *A. oryzae* on the differences in leukocyte populations and the actual mechanism behind these differences would also be beneficial. Finally, using this supplement, research into the effects on rumen microbial population and diversity is important, especially with regards to volatile fatty acid production.

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