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Effects of resistant starch in milk replacer on health and performance in neonatal holstein heifer calves

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EFFECTS OF RESISTANT STARCH IN MILK REPLACER ON HEALTH AND
PERFORMANCE OF NEONATAL HOLSTEIN HEIFER CALVES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program in
the School of Animal Sciences

By
Bethany Leann Fisher
B.S., Southern Arkansas University, 2007
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Abstract

Forty-two female Holstein calves were assigned to one of three treatments at d 2 of age to study the effects of adding resistant starch (RS) to the milk replacer on health and performance. Treatments were control (0g RS), 4g RS, or 8g RS mixed into the reconstituted milk replacer. Calves were housed in individual calf hutches and fed milk replacer once daily until d 42 of age. Water and an 18% crude protein calf starter were offered *ad libitum* beginning d 3 throughout the duration of the 56 d trial. Calves remained in their hutches until d 56 of age to determine immediate postweaning performance. Body weights (BW) were measured at birth and d 7, 14, 28, 42, and 56 d of age. Withers height (WH), hip height (HH), and hip width (HW) were measured on d 7, 14, 28, 42, and 56 d of age. Feed intake, body temperatures, and fecal scores were recorded once daily through d 56. On d 14, 28, 42, and 56, fecal samples were collected for analysis of pH and short chain fatty acids (SCFA), and blood was collected for analysis of plasma urea nitrogen (PUN) and total protein (TP). The PUN and TP were within normal ranges suggesting that there were no major metabolic problems. There was no effect ($P>0.05$) of treatment on BW, HH, HW, WH, or body temperatures. There was a treatment by week interaction ($P<0.01$) and a week effect ($P<0.01$) for grain intake, with all calves increasing intake throughout the duration of the study. There was a treatment by week interaction ($P<0.01$) for fecal scores. All calves had lower ($P<0.01$) fecal scores at the end of the study compared to the beginning. Fecal pH increased as calves aged ($P<0.01$). There was a treatment by week interaction ($P<0.05$) for propionate concentration in the feces. Propionate concentrations decreased ($P<0.01$) until weaning at week 6 for all treatments while calves consuming 4g RS had higher ($P<0.05$) concentrations compared to those consuming 8g RS over the entire trial. Acetate, butyrate, and total SCFA concentrations all decreased ($P<0.01$) for all calves until

weaning at week 6. Incorporation of RS in the milk replacer of calves did show changes in fecal SCFA at 2 and 4 weeks of age. However, incorporation of RS in milk replacer had no overall treatment effects on health and performance of neonatal dairy calves.

Chapter 1

Introduction

The cost incurred to raise a replacement dairy heifer from birth to weaning is the highest cost associated with that animal. Proper management of these animals early in life will affect their future performance. With 80.7% of producers raising their own replacements in 2006 (USDA, 2007), researchers and extension specialists are often asked how to reduce costs while following good management practices during this crucial time in the animal's life.

A common practice is the feeding of milk replacer as an economical substitute for whole milk. According to the National Dairy Herd Evaluation Project, 70.2% of United States dairy operations use milk replacer for feeding neonatal calves prior to weaning (USDA, 2007). Since milk replacers became commercially available in the 1950's several changes and improvements have been implemented. One of the more controversial yet economically important improvements made has been the addition of antimicrobials to promote more efficient growth and for the prevention, treatment, and control of bacterial enteritis. However, with the inclusion of antimicrobials, there is a concern of bacterial resistance to these compounds.

In June of 2010 the FDA published a draft guidance informing the public of their concerns on the routine use of medically important antimicrobial drugs in feed of food-producing animals. The report stated that "antimicrobial resistance, and the resulting failure of antimicrobial therapies in humans, is a mounting public health problem of global significance." The FDA finds the use of medically important antimicrobial drugs in food producing animals for use as growth promotants and improved feed efficiency an injudicious use. However uses associated with the treatment, prevention, or control of specific diseases including incorporation in feed and water are considered to be necessary uses for assuring the health of food producing

animals (US-DHHS, 2010). This being said some drugs have been banned, others limited in uses, and stricter guidelines are being put in place for any new drugs approved for market. Those already on the market are being reevaluated to determine their effect on antimicrobial resistance in humans.

The federal government is looking to researchers and extension specialists to find a substitute for prophylactic use of antibiotics in milk replacers for neonatal calves. Many pre- and probiotic additives are already on the market each showing variable results in many species. One product being tested for its prebiotic potential is resistant starch (RS). Many of the beneficial effects of RS on large bowel function appear to be exerted through short chain fatty acid (SCFA) production from bacterial fermentation (Topping et al., 2003). These SCFA may lower the gut pH to levels below those at which pathogens are able to effectively compete (Manning and Gibson, 2004), giving rise to beneficial bacteria and improving the overall health of the animal. RS in the forms of raw potato starch or high amylose maize starch has shown to influence fecal production of total SCFA as well as the individual concentrations of acetate, butyrate, and propionate (Bird et al., 2009; Henningsson et al., 2003; Kleessen et al., 1997; Noakes et al., 1996; Topping et al., 1997; van Munster et al., 1994). In humans the primary mode of action of RS in the colon is through fermentation, stimulation of bacterial growth, and SCFA production (Cummings, 2001). Little research has been conducted on the effects of RS on health and performance of neonatal dairy calves.

The objective of this experiment was to determine the effects of feeding of RS on health and performance of preruminant calves when included in the milk replacer.

Chapter 2

Review of Literature

Background

Heifer rearing represents about 20% of the total operating expenses on dairy farms, making it the second largest expense after feed costs (Heinrichs, 1993). Every heifer calf born on a dairy farm represents an opportunity to maintain herd size, improve herd genetics, and improve economic returns to the farm. In the period from birth to weaning, the calf is more susceptible to many diseases, more sensitive to environmental stresses, and more responsive to management changes. Neonatal calf diarrhea is an important cause of morbidity and mortality in young ruminants (Gaggia et al., 2010). In a recent survey, it was reported that 7.8% of dairy heifers died prior to weaning and more than half died as a result of scours or bacterial enteritis (USDA, 2007).

Farm animals are often subjected to physiological, psychological, and environmental stressors leading to dysfunction of the intestinal barrier and an increase in intestinal permeability resulting in an imbalance in the intestinal ecosystem, including microbial composition and susceptibility to enteric pathogens (Gaggia et al., 2010; Gareau et al., 2009). Beneficial bacteria, such as *Lactobaccilli* and *Bifidobacteria*, have been shown to decrease when stressing factors occur (Si et al., 2004). Since the most studied external factor that influences establishment of the intestinal microbiota is diet (Mackie et al., 1999), methods to increase the concentrations of these beneficial bacteria through diet could give producers an alternative to antimicrobial use.

Immune Development

At birth, the newborn calf has a developing immune system and an underdeveloped digestive system which require proper nutrition and management to maximize development and

ensure that the calf has the best possible chance of survival. The main objective during this period is to maximize growth and minimize disease outbreak.

Newborn calves are born agammaglobulinemic, meaning they do not have measureable concentrations of serum immunoglobulins (Ig) critical to their health and survival (Redman, 1979). The calf must obtain Ig within the first 24 hours of life, the period of macromolecular transport. Ig proteins are absorbed through the small intestine into the lymphatic system and further into the blood to become antibodies needed to protect the calf against environmental pathogens (Kruse, 1970). First milking colostrum is markedly higher in solids, Ig, fat, protein, and vitamins, than compared to whole milk (Foley and Otterby, 1978). When given in sufficient quantities, the high levels of Ig found in colostrum provide passive immunity to the newborn calf enabling it to fight off infections during the first few weeks of life (Redman, 1979).

Colostrum contains 3 major Ig proteins that provide immunity to the calf: IgG, IgA, and IgM. Of the three, IgG is of greatest concern in the first 24 hours of life. Matte et al. (1982) described the process as the entire protein molecule, without digestion altering its form or capabilities, passes through the cells on the mucosal epithelial membrane of the small intestine by pinocytosis and passive transfer into the lymphatic system. Later, the Ig is transferred via the thoracic lymph duct and anterior vena cava to the blood where they become active antibodies (Matte et al., 1982). The IgG then assist in reducing the incidence and severity of many different types of gastrointestinal infections, including *Escherichia coli*, rotavirus, and *Cryptosporidium parvum*. In order to assure maximum absorption of IgG, calves must receive 3-4 liters of high quality colostrum within 3-4 hours of birth.

Development of Digestive System

Once calves are no longer fed colostrum and transition milk, most producers begin feeding a commercial milk replacer. When a calf consumes colostrum or milk replacer, the liquid feed bypasses the rumen and goes directly into the abomasum via the esophageal groove. It then enters the abomasum which bears many similarities to that of a monogastric stomach. From the abomasum, the remaining components of the milk replacer enter the intestinal tract with the colon being the final stop before excretion.

The concept of the gastrointestinal (GI) tract as an ecosystem is based on the interactions among the resident assemblages of microorganisms, the structural and functional characteristics of the GI tract and the responses to dietary inputs (Buddington, 2009). At birth the neonatal calf's digestive system functions similar to that of a monogastric animal. The GI tract has been described as a physical barrier that is composed of epithelial cells lining the digestive tube, tight junctions that bind them together, and a chemical barrier which consists of secretions that can influence epithelial cells and maintain barrier function (Dubert-Ferrandon et al., 2008). Takahashi and Kiyono (1999) described the intestinal tract as the largest immunological organ of the body, while Brouns et al. (2002) concluded it was the organ with the greatest surface area and metabolic capacity. The presence of a balanced gut microflora is required for this system to function efficiently.

Mackie et al. (1999) noted that the microbial succession of the GI tract in the first few weeks of life of the preruminant calf is strikingly similar to that of a monogastric newborn. During the birth process and shortly thereafter, microbes from the mother and surrounding environment colonize the GI tract of the infant or neonatal calf (Mackie et al., 1999). The dairy calf is removed from its mother shortly after birth and fed the mother's colostrum. During this

process the calf is in constant contact with sources of bacteria from the environment, handler, and in the colostrum resulting in the initial colonization of the GI tract.

The large intestine is the primary site of microbial colonization and is characterized by large numbers of bacteria and relatively high SCFA concentrations (Mackie et al., 1999). Cummings (1997) stated in the human the colon, along with its bacterial microflora, is an important organ that provides a variety of functions, such as digestion, fermentation, immunological and protective actions, as well as detoxifying functions, which are essential to the whole organism. According to Roberfroid (2008) the gut microflora appears to play important nutritional and physio-pathological roles such as the prevention of gut colonization by potentially pathogenic microorganisms by efficiently outcompeting invading pathogens for ecological niches and metabolic substrates. Callaway et al. (2008) considers the microbial population of the intestinal tract a complex natural resource that can be utilized in an effort to reduce the impact of pathogenic bacteria that affect animal production and efficiency.

Along with modulation of the immune system, the microflora also provides important sources of energy for the cells of the gut wall through fermentation of carbohydrates to SCFA (Roberfroid, 2008). Macfarlane et al. (2008) found SCFA production to be one of the most important physiological processes mediated by colonic microorganisms. The three main SCFAs produced from this fermentation are acetate, butyrate, and propionate. These SCFAs have been shown to affect colonic epithelial cell transport, colonocyte metabolism, growth and differentiation, provide energy to various organs of the body, and aid in reducing the severity of diarrhea (Cummings, 1995; Cummings and MacFarlane, 1997; Nugent, 2005). Butyrate has been shown to induce cell growth and increase absorptive surface area of the gut and is metabolized by the colonic epithelium, changing the microflora of both the small and large intestines

(Cummings and Macfarlane, 1997; Hawthorne and Abrams, 2007). Propionate is shown to improve large bowel circulation thereby stimulating epithelial proliferation and helping to maintain epithelial integrity (Annison and Topping, 1994; Sakata, 1989). While found in the greatest quantities in the colon, acetate exerts its actions on other organs of the body and is used in the formation of butyrate. Increased SCFA concentrations and lowered pH is thought to prevent the overgrowth of pH-sensitive pathogenic bacteria (Topping and Clifton, 2001) allowing proliferation of beneficial bacteria and thereby improving the overall health of the GI system of the animal.

Antimicrobials

In an effort to maximize health and performance of neonatal dairy calves, antimicrobials have been incorporated into the milk replacer for more than 50 years. Antimicrobials are defined as any substance that kills or inhibits the growth of microorganisms. Antibiotics are substances produced by microorganisms that kill or inhibit the growth of other microorganisms (USDA, 2010). These two terms are often used interchangeably.

The use of medicated milk replacers have become a common practice of dairy producers (Vissek, 1978). Antimicrobials are used in feedstuffs of young animals for several reasons. At subtherapeutic levels antimicrobials have been shown to prevent, control, and treat enteric infections; while improving growth and overall feed efficiency at a critical time in the animal's life. In 2006 it was reported that 49.9 % of all heifers received medicated milk replacers at some point prior to weaning (USDA, 2007).

Donovan et al. (2002) reported that the addition of antibiotics to the milk replacer, starter, or water of young calves improves feed efficiency, growth, starter consumption, and phagocytic efficiency. Morrill et al. (1977) reported that the inclusion of antibiotics in the milk of calves

resulted in increased gain as well as feed consumption even when there was no presence of digestive disease. Berge et al. (2005) reported that calves fed a medicated milk replacer had decreased overall morbidity and increased weight gain compared with calves fed a non-medicated milk replacer. Losinger et al. (1995) reported a reduced shedding of enteropathogens, while both Quigley et al. (1997, 2002) reported a reduction in the severity and length of scouring, improved body weight gains, and improved overall efficiency of calves fed milk replacer supplemented with antibiotics.

In 2006, chlortetracycline and oxytetracycline in combination with neomycin, decoquinat, and lasalocid were the only approved antimicrobial agents that could be used in medicated milk replacers (USDA, 2007). It was reported that 49.5 % of operations used oxytetracycline in combination with neomycin in medicated milk replacers, and oxytetracycline and decoquinat were fed on nearly one of five operations (USDA, 2010).

Development of Antimicrobial Resistance

Dibner and Richards (2005) noted that pathogens, such as *Campylobacter* and *Salmonella* can be transmitted along the food chain and can be a source of human illness. Feeding even subtherapeutic levels of antibiotics over a long period of time could result in the intestinal bacteria becoming resistant to the antibiotics and when slaughtered, these resistant bacteria can enter the human food chain and cause detrimental illnesses by pathogens that are resistant to normal antibiotic therapies.

It was first documented in 1963 when increased levels of resistance were observed in a particular strain of *Salmonella typhimurium* in several British feedlots. Over a three year period several resistant isolates were subsequently identified (Dewey et al., 1997). Kaneene et al. (2008) reported that feeding non-medicated milk replacer had an increase in antimicrobial

susceptibility in *E. coli* and *Salmonella*, while feeding medicated milk replacer was associated with decreased susceptibility of *E. coli* and *Salmonella* from fecal and calf pen samples.

Langford et al. (2003) found that resistance of gut bacteria to antibiotics increased with increasing concentrations of penicillin in milk fed to dairy calves. However, Berge et al. (2005) concluded that removal of antibiotics from milk replacers may have a significant negative impact on calf health in the absence of adequate passive transfer of immunity.

Antimicrobial Resistance

Antimicrobial efficacy or the lack of efficacy of a drug for treatment of a disease agent for which the drug was previously effective was recognized soon after the widespread use of antibiotics began (Kaneene et al., 2008). One study estimated that the direct hospital cost of managing antibiotic resistance in the United States is \$100 million to \$10 billion per year, and the Office of Technology Assessment estimated in 1992 that the minimal hospital cost of 5 types of nosocomial infection due to antibiotic resistance were \$4.5 billion per year (US Office Tech, 1995). Since then several health and medical organizations have been investigating the possibility that the subtherapeutic use of antimicrobials could be increasing the level of antibiotic resistance in not only animals but humans as well. Reports by Fey et al. (2000) and Tollefson et al. (1999) suggest that continued use of antimicrobials in animal agriculture may contribute to increased risk of antibiotic-resistant bacteria of medical importance. Several products have been tested to determine efficiency of reducing bacterial enteritis and improving feed efficiency while recognizing that a properly functioning intestinal tract can greatly reduce the prevalence of enteric infections in young calves.

Alternatives

Although the use of antibiotics for removal of undesired species has been a mainstay for managing the GI tract bacteria, concerns about bacterial adaptation, development of antibiotic resistance, and destabilization of the commensal assemblages have stimulated the search for alternative strategies (Collins et al., 2009). The use of non-antibiotic feed additives is becoming increasingly popular to maintain or stimulate intestinal and systemic immunity and maintain high levels of animal performance. In order for a product to reduce enteric disease it must be able to survive processing, storage, and handling of animal feeds, as well as, being able to survive the environment of the rumen and/or abomasum of the animal and not be degraded by intestinal enzymes allowing it to then act while in the intestinal tract (Roberfroid, 2008). Donovan et al. (2002) provided evidence that growth and performance of calves receiving probiotics, and the prebiotics, allicin, and fructooligosaccharides (FOS) were equivalent to that of calves fed antibiotics during the first 5 weeks of life.

Mechanisms

Mechanisms by which probiotic and prebiotic supplements affect the micro-ecology of the intestinal tract are not well studied, but at least 3 mechanisms have been identified that could have significance in neonatal calves. Antibacterial agents that are produced and secreted by probiotic organisms may have an inhibitory effect on growth of pathogenic microflora. The stimulation of immune responses may also suppress potential pathogens. Finally, specific competition for adhesion receptors to the gut epithelium may allow lactic acid bacteria and *Bifidobacteria* to occupy the niche normally required by toxin-producing organisms for colonization. A basic mechanism involved in limiting intestinal pathogens is the competition for nutrients and attachment sites on the intestinal mucosa allowing beneficial bacteria to occupy

these attachment sites and increase fermentation and SCFA production. The increased production of short chain fatty acids results in a lower intestinal pH and thereby prevents the proliferation of some disease causing pathogens that are unable to withstand a more acidic environment (Rowland and Gill, 2008).

Probiotics

The concept of probiotics goes back more than 100 years to Elie Metschnikoff who proposed that bacteria in fermented milk products may be able to control bacterial fermentation in the intestinal tract of men and are thus health promoting (Metschnikoff, 1907). Probiotics are dietary supplements containing bacteria or yeast, and the use of these is based on the concept that feeding “beneficial” microbes to livestock subjected to stress conditions may prevent establishment of undesirable microorganisms while helping to re-establish normal gut microflora, thus benefiting animal health and performance (Gaggia et al., 2010). Probiotics act mainly via modifications of intestinal bacterial populations and their effectiveness depends on the microbial status of a group of animals and the individual animal.

Abe et al. (1995) reported the addition of probiotics to the diets of preweaned calves increased body weights and decreased incidence of scours. Agarwal et al. (2002) found a reduction in the incidence of diarrhea in calves fed milk fermented with either lactic acid bacteria, or *L. acidophilus* 15 or *S. cerevisiae* NCDC49. Adams et al. (2008) found an improvement in weight gain and rumen development in young calves supplemented with bacterial and yeast strains, in contrast to Jenny et al. (1991) who reported no significant effects on growth and health of neonatal calves when probiotics were included in the milk replacer. However, a shortcoming of probiotics is that only a small proportion of ingested organisms reach the colon intact due to passage through the stomach and small intestines (Topping et al., 2003).

Prebiotics

Unlike the situation with probiotics, where allochthonous microorganisms are being introduced into the gut, and have to compete against established colonic communities, prebiotics target the bacteria that are already commensal to the large intestine (MacFarlane et al., 2008). According to Walker and Duffy (1998) the development of prebiotics came from the idea that non digestible food ingredients are selectively fermented by one or more bacteria known to have positive effects on gut physiology thus giving the bacteria a proliferative advantage over less beneficial or potentially harmful bacteria. Menne et al. (2000) stated that the key criterion for a prebiotic effect is the demonstration of the selective stimulation of growth of one, or a limited number of potentially beneficial bacteria in the complex fecal microbiota following the consumption of a particular food. Research has indicated that the methods by which prebiotics can exert their effects on the immune system and attenuate inflammation in the colon include increased short chain fatty acid production and increased immunogenic bacteria which contribute to the establishment of a “healthier” microbiota, such as *Lactobacilli* and *Bifidobacteria*. These organisms become predominant and exert possible health-promoting effects at the expense of more harmful species (MacFarlane et al., 2008; Gaggia et al., 2010).

There are several prebiotics on the market including mannanoligosaccharides (MOS), fructooligosaccharides (FOS), and inulin that have shown beneficial effects on the intestinal health of preweaned monogastrics. Dietary intervention that increases the amount of SCFAs in the colon is thought to be beneficial to gut health, hence SCFA are commonly used as markers of fermentation and colonic health (Nugent, 2005). Oligosaccharides are a class of carbohydrates that are not absorbed or digested in the small intestines of man and animals and thus reach the colon unaltered. Oligosaccharides resemble fimbriae and serve as a “decoy” attachment for

bacteria (Hill et al., 2009). Bacteria that attach to oral oligosaccharides do not attach to the intestinal epithelium, reducing the risk of infection (Hill et al., 2009). Fructooligosaccharides (Quigley et al., 2002) and MOS, derived from mannan yeast cell walls, stimulate growth of beneficial bacteria in the GI tract by blocking colonization of pathogens by providing an alternative attachment site for the pathogen rather than the intestinal mucosa and are thereby excreted in the feces (Quigley et al., 2002; Spring et al., 2000). Brouns et al. (2002) noted recent indications that the regular consumption of certain subclasses of highly fermentable dietary fiber sources result in gut associated immune and flora modulation as well as significant production of SCFA. Gaggia et al. (2010) found the use of prebiotics in animal production, as a possible alternative to antimicrobial growth promoters, has shown contradictory results, while their use in the modulation of the gut microbial equilibrium is worthwhile.

Recent research suggests that MOS plays a role in influencing intestinal microflora and SCFA production, and also may have a direct effect on the gut associated immune response (Forchielli and Walker, 2005). Several reports of MOS supplementation in milk replacers of dairy calves have shown higher overall probabilities of normal feces throughout the studies, but no significant difference in growth, feed efficiency, or average daily gain (ADG) compared to calves not receiving MOS (Heinrichs et al., 2003; Hill et al., 2008; Hill et al., 2009; Terre et al., 2006). However, Heinrichs et al. (2003) reported a more rapid recovery from scouring in calves fed MOS compared to the control group. Terre et al. (2006) found calves supplemented with MOS tended to consume more grain prior to weaning and consumed greater quantities of starter in the week following weaning compared to the control calves; while Fairchild et al. (2001) reported improved health and growth of poultry when challenged with *E. coli* and fed Bio-Mos. A reduction of nearly 1 pH unit in fecal samples of humans has been reported after consuming

diets supplemented with oligofructose (Menne et al., 2000). In young piglets oligofructose has shown a reduction in the severity and duration of dysentery (Oli et al., 1998; Russel et al., 1995). Berg et al. (2005) reported a decrease in fecal pH and an increase in total fecal SCFA as the level of FOS supplementation increased in yearling horses. FOS inclusion in human diets appears to result in fecal bulking and selective stimulation of *Bifidobacteria* growth in the colon (Van Loo et al., 1999). Brown et al. (1998) found that pigs fed diets based on human foods and supplemented with FOS and RS resulted in an increase in fecal butyrate that was greater than groups fed either FOS or RS separately. Similarly Topping et al. (2003) reported that pigs fed FOS and RS were able to maintain bacterial colonization after probiotic consumption had ceased.

Resistant Starch

Starch is a complex carbohydrate containing amylose and amylopectin (Tulley et al., 2009). Amylopectin is composed of glucose molecules joined by α -(1-4) linked chains with α -(1-6) linked branch points. The amylose component consists of linear α -(1-4) linked chains and is referred to as the resistant portion. The linearity of the amylose portion allows it to fold back on itself and stack tightly avoiding digestion and absorption in the small intestine and to be fermented in the cecum or large intestine (Tulley et al., 2009). Topping et al (2003) stated that RS, as a high amylose starch, is a prebiotic that exerts its actions through adhesion of bacteria to the granule surface. It has been noted that high amylose RS granules develop a particular etching pattern in their passage through the upper gut of both humans and pigs (Topping et al., 1997). These etchings result in surface pitting that could improve the viability of probiotics and beneficial bacteria, particularly *Bifidobacteria*, both supplemented and already present in the large intestine and colon by providing a surface for physical adhesion (Brown et al., 1998;

Nugent, 2005). Once RS reaches the colon, fermentation is relatively slow (Brouns et al., 2002) and should result in an increased production of SCFA.

Complete fermentation by microflora, reduction in intestinal pH, selective utilization by *Lactobacilli* and *Bifidobacteria*, reduction of intestinal pathogen levels, reduction in symptoms associated with diarrhea, and stimulation of the immune system are just a few of the functional properties of RS described by Brouns et al. (2002). Two major effects of resistant starch are a bulking effect similar to that seen in fermentable fiber and fermentation to short chain fatty acids in the gut (Englyst et al., 1992; Nugent, 2005). Interest in the potential of RS as a prebiotic grew out of animal and human studies where consumption of high-RS foods and ingredients led to a time dependent shift in fecal and large-bowel SCFA profiles (Topping et al., 2003; Zhou et al., 2008) and a reduction of pH in the large intestine (Le Leu et al., 2002; Younes et al., 2001; Zhou et al., 2008). This SCFA production has also been speculated as a cause of satiety due to expansion of the abdomen from gas production providing a full feeling (Tulley et al., 2009). Metabolic end-products, such as acids excreted by microorganisms, may lower the gut pH to levels below those at which pathogens are able to effectively compete (Manning and Gibson, 2004).

Consumption of resistant starch has been shown to assist in recovery from infectious diarrhea in man and animals (Topping et al., 2003). Keenan et al. (2006) conducted several studies in rats that indicated that fermentable resistant starch has possible physiological benefits, including lowering abdominal fat, as well as, increasing feed intake in order to compensate for a dilution of metabolizable energy with non-digestible fermentable or non-fermentable fibers. In rats fed RS an increase in fermentation and a decrease in cecal pH (Tulley et al., 2009) and an increase in both fecal acetate and total SCFA concentrations (Kleessen et al., 1997) have been

reported. Kleessen et al. (1997) did not see a difference in intake, body weight gain, or growth in rats fed RS compared to the controls. Noakes et al. (1996) reported increased fecal butyrate levels in humans consuming RS, while Phillips et al. (1995) reported an increase in both acetate and butyrate concentrations in the feces of humans after RS supplementation. A reduction in colonic pH after RS supplementation has also been noted in several studies, both human and rat, potentially resulting in rapid SCFA absorption (Noakes et al., 1996; Phillips et al., 1995; Le Leu et al., 2002).

Chapter 3

Materials and Methods

Animals/Treatments

Forty-two female Holstein calves were utilized to determine the effects of inclusion of two levels of resistant starch on growth and performance from birth to 56 d. All calves were born at the LSU Agricultural Center Research and Teaching Dairy Farm, Baton Rouge, LA, between August 2009 and February 2010 and housed there for the duration of the experiment. The experimental protocol was approved for use by the Institutional Animal Care and Use Committee (IACUC) of the LSU Agricultural Center. Calves were removed from their dams at birth, weighed, orally vaccinated against Rotavirus and Coronavirus (Calf Guard, Pfizer Animal Health, Lenexa, KS) and placed in a hutch. Navels were disinfected with a 4% iodine solution, and all calves received 3-4 liters of high quality colostrum within 3 hours of birth. All colostrum had ≥ 50 mg/dl IgG concentration as measured by a colostrometer. Individual hutches measured 2.5 m² and connected to a wire enclosure measuring 2.3 m². All hutches were located on a bed of scallop rock approximately 8 cm thick and arranged so calves could not come in physical contact with each other.

Calves were bottle-fed colostrum and transition milk collected from their dams for d1 and 2 of life. Calves were then randomly assigned to one of three treatments by birth date: control calves (0g RS) receiving no RS; calves receiving 7.1g of Hi-maize corn starch (56% RS, delivering 4g RS/calf/day) (4g RS); and calves receiving 14.3g of Hi-maize corn starch (56% RS, delivering 8g RS/calf/day) (8g RS). On d 3, calves were offered milk replacer (MR) (20% protein, 20% fat; Nutra Blend LLC, Neosho, MO) (Table 1) containing decoquinate at 10% of their birth weight and mixed to contain 15% solids. RS was mixed in MR immediately before

feeding. Calves were offered MR once daily at 0630 from d 3 until abrupt weaning at d 42.

Refusal of MR was weighed and discarded.

All calves were fed an 18% crude protein (CP) calf starter (Table 2) *ad libitum* from d 3 until d 56 of age. Fresh starter was offered daily and daily refusal was recorded. Water was offered *ad libitum* beginning on d 3.

Table 1: Milk Replacer Nutrient Analysis

Active Drug Ingredient: Decoquinat.....45.4g/Ton (22.7mg/Lb)			
Guaranteed Analysis	Control	Low	High
Crude Protein , Minimum	20.00%	20.00%	20.00%
Crude Fat, Minimum	20.00%	20.00%	20.00%
Crude Fiber, Maximum	0.15%	0.15%	0.15%
Calcium, Minimum	0.50%	0.50%	0.50%
Calcium, Maximum	1.00%	1.00%	1.00%
Phosphorus, Minimum	0.60%	0.60%	0.60%
Vitamin A, Minimum	35,000 IU/Lb	35,000 IU/Lb	35,000 IU/Lb
Vitamin D ₃ , Minimum	7,500 IU/Lb	7,500 IU/Lb	7,500 IU/Lb
Vitamin E, Minimum	150 IU/Lb	150 IU/Lb	150 IU/Lb
Hi-maize Corn Starch (56% RS)	0g	7.1g (4g RS)	14.3g (8g RS)

Table 2: Calf Starter Composition and Chemical Analysis

Ingredients	% As Fed
Cracked Corn	21.06
Magnesium Oxide	0.25
Calcium Carbonate	0.15
Dried Distiller Grain with solubles	5.00
Soybean Meal 48	11.00
Protein Pellets ^a	10.00
Oats Crimped	17.50
Molasses	5.00
Friends Hi-Fat 14-5 ^b	3.00
Chemical Analysis	
Dry Matter (%)	92.75
Crude Protein (CP), (% DM)	18.50
Acid Detergent Fiber (ADF)	10.20

^aProtein Pellets are 30.2% CP (as fed) and 2.01% Crude Fat (as fed).

^bFriends Hi-Fat 14-5 is 14% CP (as fed) and 5% Crude Fat (as fed).

Collection Procedures

Body weights were recorded at birth and at 7, 14, 28, 42, and 56 d of age. Wither height (WH), hip height (HH), and hip width (HW) were measured at 7, 14, 28, 42, and 56 d of age.

Fecal scores and body temperatures were recorded once daily at 1100. Fecal scores (fluidity) were recorded according to Larson et al. (1977) using a 1 to 4 scale as follows: 1 = normal, 2 = soft, 3 = runny, and 4 = watery. Fecal samples were collected approximately 4 hr post-feeding for pH and short chain fatty acid (SCFA) analysis at 14, 28, 42, and 56 d of age. Fecal samples were diluted using 1 g of feces to 4.5 mL of distilled water, pH was determined, and 0.5 mL of phosphoric acid (20% w/v) was added. Samples were protected from light and stored frozen (-20° C) until analysis. For total SCFA analysis, a 4 mL sample of fecal fluid was mixed with 1 mL of 25% (wt/wt) meta-phosphoric acid containing 10 g/L 2-ethylbutyric acid, as an internal standard. The mixture of feces and meta-phosphoric acid was centrifuged at 30,000 x g for 25 min. Concentrations of individual SCFA were measured by gas liquid chromatography (GLC) using a Shimadzu GC2010 equipped with a 15-m EC-1000 column with an internal diameter of 0.53 mm and a film thickness of 1.2 µm (Alltech Associates, Inc.; Deerfield, IL). The reagent preparation procedure and temperature gradient for SCFA analysis was adapted from Grigsby et al. (1992) and Bateman et al. (2002), respectively (Appendix A).

Blood was collected at 24 h and 14, 28, 42, and 56 d of age via jugular venipuncture. Blood was collected for IgG concentration at 24 hr of age; and Total Protein (TP), Plasma Urea Nitrogen (PUN), and hematocrit at 14, 28, 42, and 56d. Blood for PUN analysis was collected in 10 mL vacutainer tubes containing sodium heparin, centrifuged for twenty minutes at 600 x g, and plasma separated, protected from light, and stored frozen (-20°C) until analysis. PUN levels for d14, 28, 42, and 56 were measured using commercial spectrophotometric kits, (Urea

Nitrogen (BUN) Berthelot/Colorimetric; Pointe Scientific, Inc., Canton, MI) (Appendix B). Blood for serum TP and IgG were collected in 10 mL vacutainer tubes containing no additive, centrifuged for twenty minutes at 600 x g, and serum was separated, protected from light, and stored frozen (-20°C) until analysis. Serum TP levels for d14, 28, 42, and 56 were measured using commercial spectrophotometric kits, (Total Protein (Biuret) Reagent Kit; Pointe Scientific, Inc., Canton, MI) (Appendix C). Serum IgG levels were measured on 24hr blood samples using a turbidimetric method (MBC QT II™ Bovine Serum IgG; Midland Bioproducts Corporation, Boone, IA) (Appendix D).

Statistical Methods and Calculations

All dependent variables were analyzed using the MIXED procedure (Littell et al., 1996) of SAS®. For all variables measured over weeks, the model included treatment, week, and treatment by week interactions as fixed effects. The random variable was calf nested within treatment. Week was the repeated term that was assumed to be correlated within calf. A compound symmetry covariance structure was assumed for the variance-covariance matrix.

All pairwise main effects were tested using Tukey's HSD test. When significant effects were noted post hoc pairwise t-test of least-squares means were used to determine significance of interactions. Effects and interactions of all measured parameters were determined to be significant at $P < 0.05$.

Chapter 4

Results and Discussion

Animal Growth

Least squares means for body weight (BW), hip height (HH), hip width (HW), and wither height (WH) are presented in Table 3 and Figures 1, 2, 3, and 4, respectively.

There was no main effect of treatment ($P>0.05$) or treatment by week interaction ($P>0.05$) on BW, HH, HW, or WH. Figures 1, 2, 3, and 4 show an increase ($P<0.05$) in BW, HH, HW, and WH over time. Studies with high amylose maize starch (HAMS) in rats (Kleessen et al., 1997) and mannanoligosaccharides (MOS) (Heinrichs et al. 2003; Hill et al., 2009; Hill et al., 2008), or fructooligosaccharides (FOS) (Hill et al., 2008) in preweaned calves reported no differences in BW, HH, HW, or WH. In chickens (Fairchild et al., 2001) and calves (Dvorak et al., 1997) supplemented with MOS, improved BW was reported.

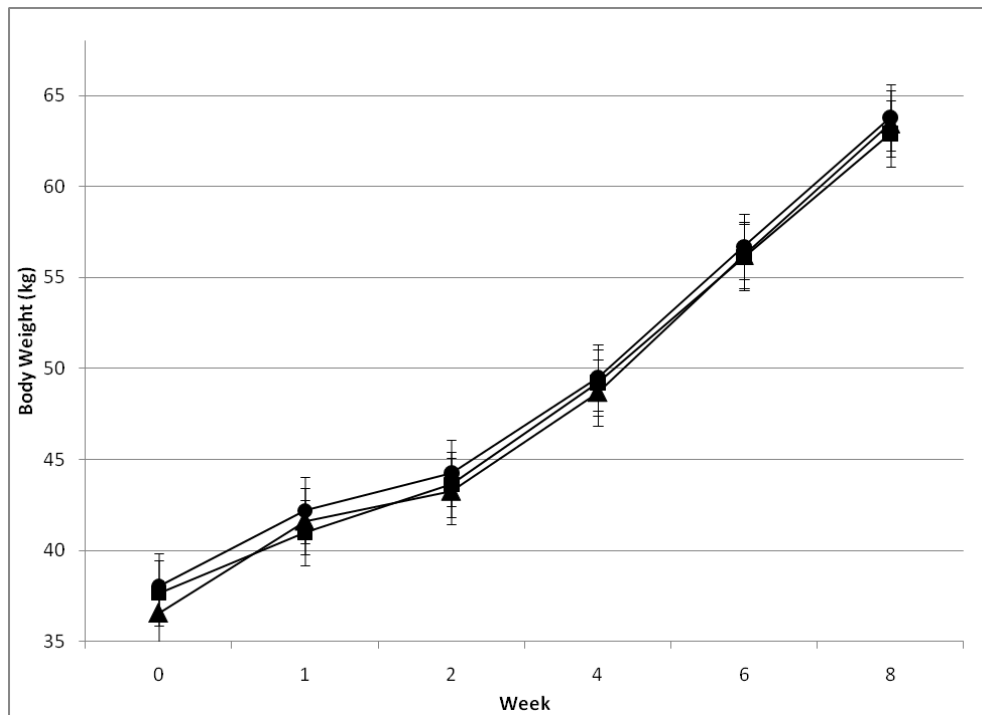


Figure 1: Least squares means for average body weight for calves fed milk replacer containing 0 (●), 4 (■), or 8g (▲) resistant starch (RS). Main effect of week ($P<0.05$).

Table 3: Least squares means for average body weight, hip height, hip width, wither height, total body weight gain, and average daily body weight gain for calves fed milk replacer containing 0, 4, or 8g resistant starch (RS).

	Treatment				
Item	0g	4g	8g	SEM ¹	P-Value
Body Weight, kg					
Birth	38.00	37.65	36.55	1.22	0.68
Weaning, wk 6	56.70	56.12	56.21	1.89	0.97
Final, wk 8	63.79	62.92	63.44	2.42	0.97
Total BW Gain, kg					
Birth to wk 6	18.69	18.53	19.67	1.28	0.80
Wk 6 to wk 8	7.10	6.80	7.23	1.23	0.97
Birth to wk 8	25.79	25.34	26.89	1.96	0.85
ADG², kg/d					
Birth to wk 6	0.45	0.44	0.47	0.03	0.80
Wk 6 to wk 8	0.51	0.49	0.52	0.09	0.97
Birth to wk 8	0.46	0.45	0.48	0.03	0.85
Hip Height, cm					
Weaning, wk 6	86.59	86.38	85.80	0.78	0.77
Final, wk 8	88.61	89.02	87.84	0.88	0.63
ADG, cm/d	0.16	0.16	0.17	0.01	0.79
Hip Width, cm					
Weaning, wk 6	19.51	19.32	19.24	0.24	0.71
Final, wk 8	20.55	20.50	20.45	0.30	0.97
ADG, cm/d	0.07	0.07	0.08	0.01	0.53
Wither Height, cm					
Weaning, wk 6	82.04	82.11	80.92	0.76	0.46
Final, wk 8	84.25	84.43	82.79	0.81	0.30
ADG, cm/d	0.17	0.19	0.19	0.01	0.48

¹SEM=Standard Error of Mean

²ADG=Average Daily Gain

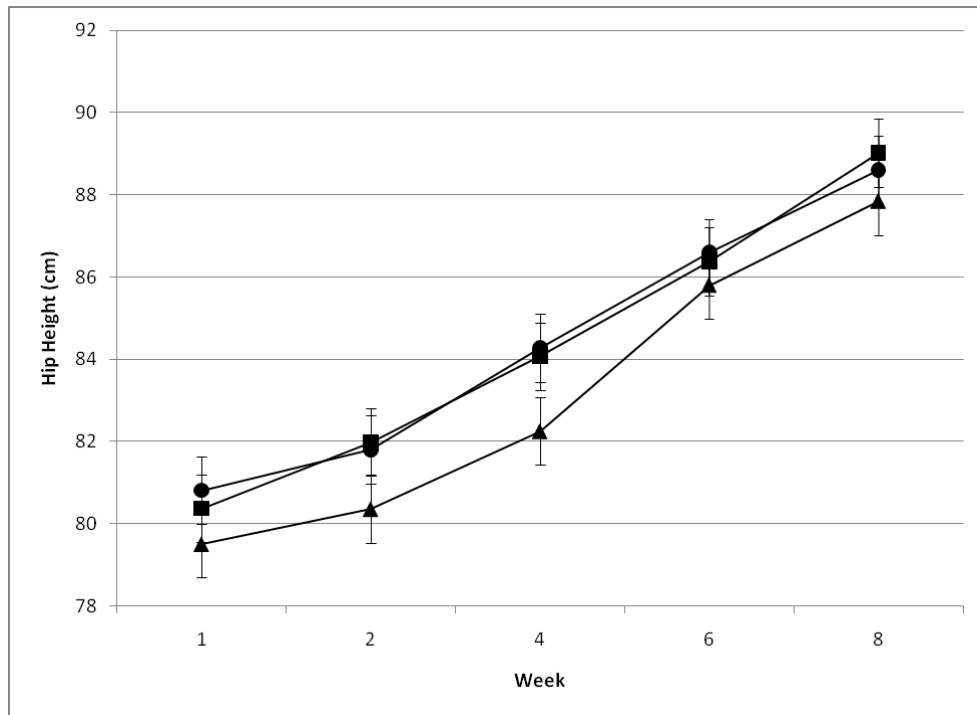


Figure 2: Least squares means for average hip height for calves fed milk replacer containing 0 (●), 4 (■), or 8g (▲) resistant starch (RS). Main effect of week ($P<0.05$).

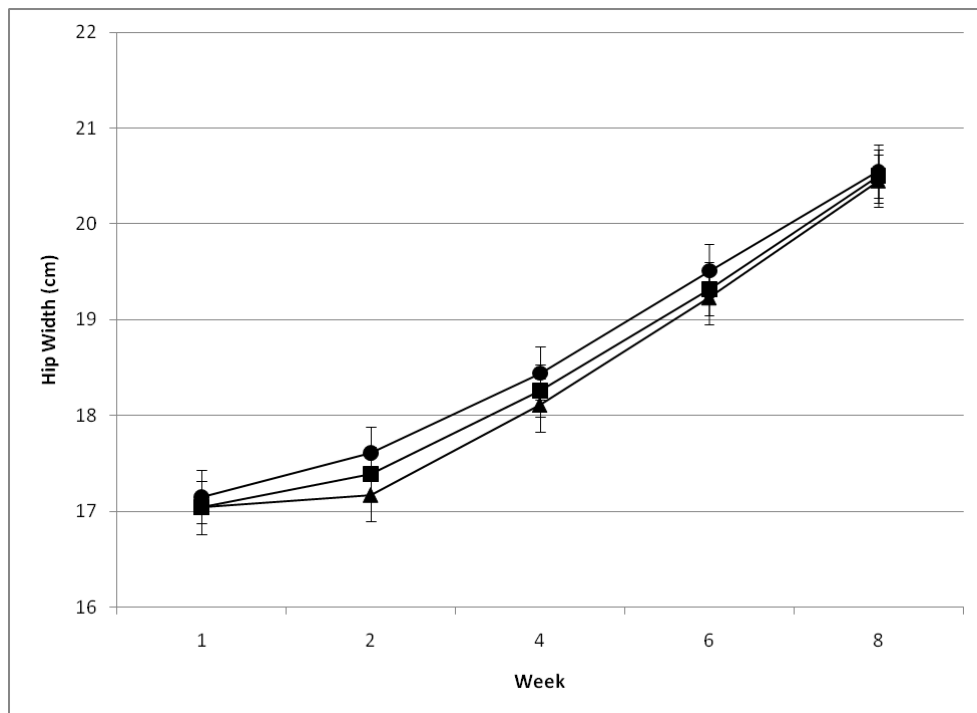


Figure 3: Least squares means for average hip width for calves fed milk replacer containing 0 (●), 4 (■), or 8g (▲) resistant starch (RS). Main effect of week ($P<0.05$).

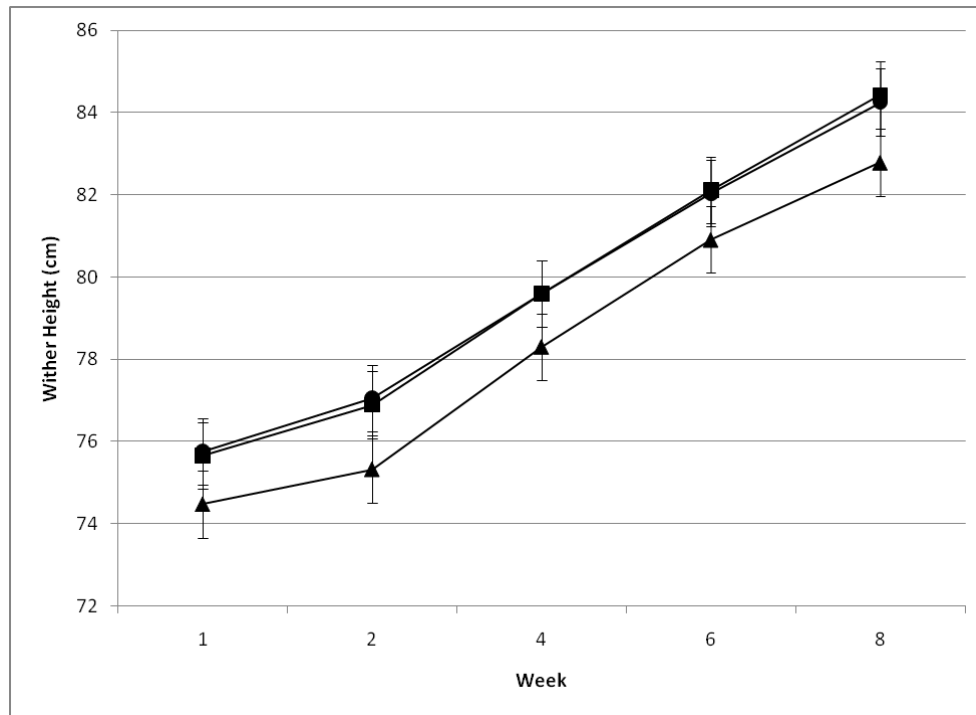


Figure 4: Least squares means for average wither height for calves fed milk replacer containing 0 (●), 4 (■), or 8g (▲) resistant starch (RS). Main effect of week ($P < 0.05$)

Health and Performance

Least squares means for starter dry matter intake (DMI), total DMI, and feed efficiency (FE) for calves are presented in Table 4 and Figures 5, 6, and 7. A treatment by week interaction ($P < 0.01$) and a main effect of week ($P < 0.01$) were observed for both starter and total DMI. Starter DMI (Figure 5) and total DMI (Figure 6) were very low in the first 2 weeks of life during which time the primary portion of the calf's diet was milk replacer. As the calf began eating starter grain, rumen development was stimulated and intake improved. Along with this increase in DMI was the increase seen previously in BW, HH, HW, and WH as the calf aged. While starter DMI was higher ($P < 0.01$) in calves consuming 0g RS compared to those consuming 4 or 8g RS at week 8, there was no main effect ($P > 0.05$) of treatment (Table 4). Kleessen et al. (1997) in rats with resistant starch (RS) supplementation found no significant effect in DMI during the

treatment periods. Some studies have reported improved intake in calves after MOS supplementation (Dvorak et al. 1997; Heinrichs et al., 2003; Terre et al., 2006). In the present study no treatment by week interaction ($P>0.05$) or main effect of treatment ($P>0.05$) was observed for FE. A main effect of week ($P<0.01$) was reported for FE (Figure 7). Feed efficiency over the course of the study did not differ among the treatment groups (Table 4). The decline in postweaning FE from 6 to 8 weeks was not significantly different and was a result of two calves fed 4g RS and one calf fed 0g RS having postweaning weight loss.

Table 4: Least squares means for average starter DMI, total DMI, and feed efficiency (FE) for calves fed milk replacer containing 0, 4, or 8g resistant starch (RS).

Item	Treatment			SEM ¹	P-Value	
	0g	4g	8g			
Starter DMI, g/d						
Week 5 to 6	540.69	507.69	493.98	55.87	0.83	
Week 7 to 8	1640.34	1456.88	1440.23	106.48	0.35	
Entire Trial	723.15	662.83	660.78	55.98	0.67	
Total DMI², g/day						
Week 5 to 6	1109.36	1068.49	1044.13	59.98	0.74	
Week 7 to 8	1640.34	1456.88	1440.23	106.48	0.35	
Entire Trial	1039.84	982.86	967.86	50.69	0.57	
Feed Efficiency³						
kg Total DMI:kg BW gain						
Week 5 to 6	2.58	2.22	2.18	0.69	0.86	
Week 7 to 8	2.10	1.74	3.28	0.69	0.86	
Entire Trial	2.28	2.38	2.09	0.19	0.55	

¹SEM = Standard Error of Means

²Total DMI = Milk Replacer +Starter DMI

³Feed Efficiency = kg of Total DMI per kg BW gain

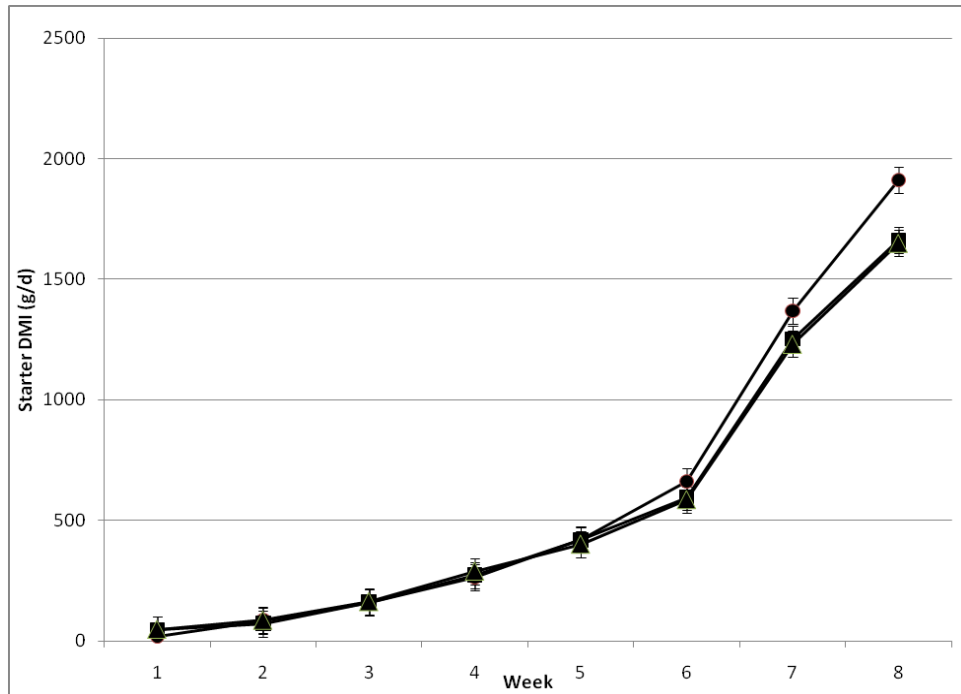


Figure 5: Least squares means for average weekly starter dry matter intake (g/d) for calves fed milk replacer containing 0 (●), 4 (■), or 8g (▲) resistant starch (RS). Main effect of week ($P<0.01$); Treatment by week interaction ($P<0.01$).

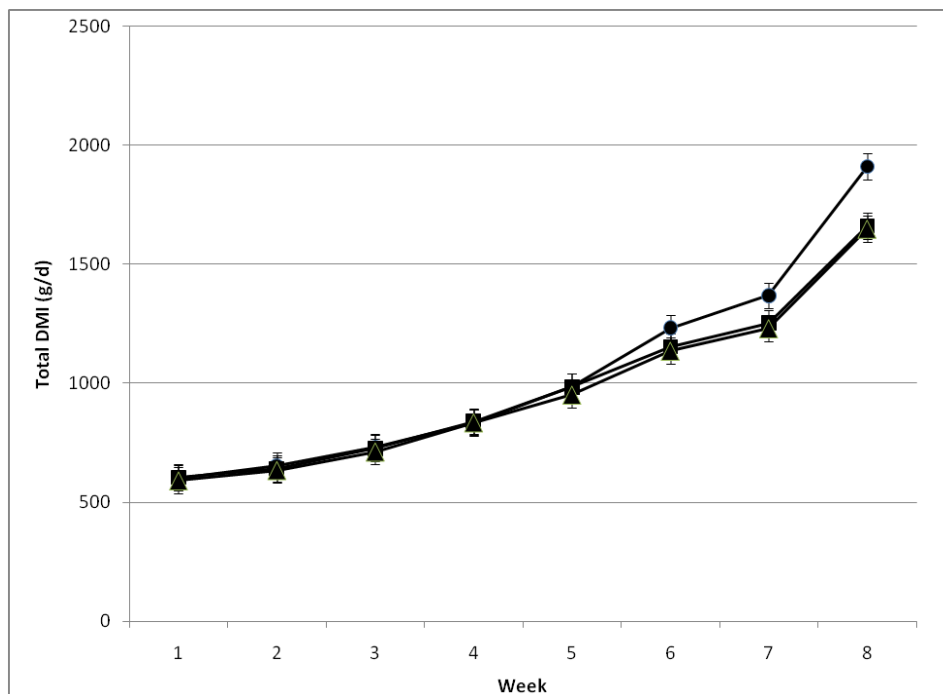


Figure 6: Least squares means for average weekly total dry matter intake (g/d) for calves fed milk replacer containing 0 (●), 4 (■), or 8g (▲) resistant starch (RS). Main effect of week ($P<0.05$); Treatment by week interaction ($P<0.01$).

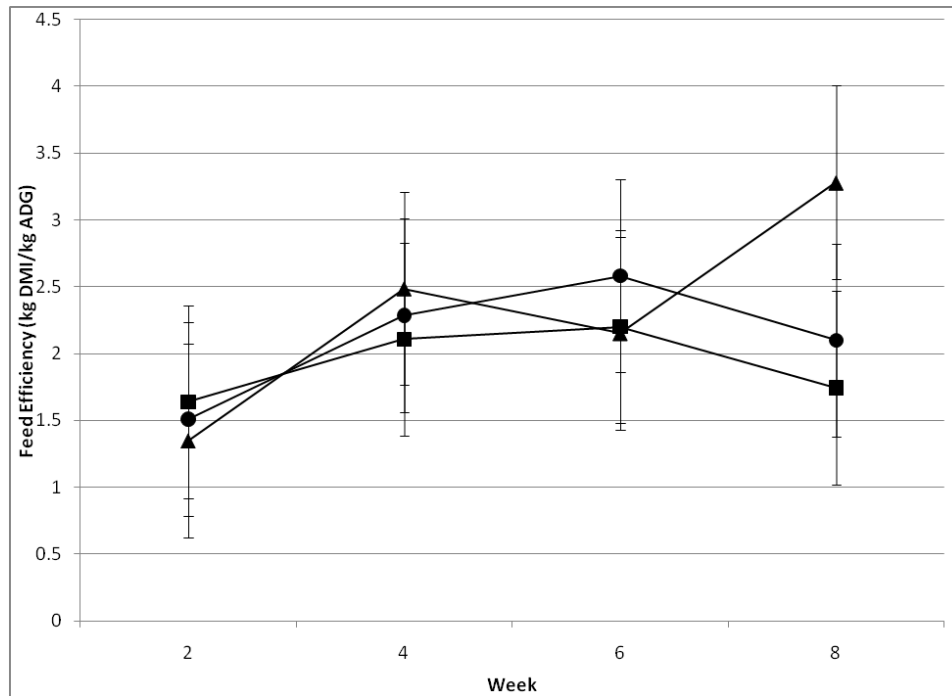


Figure 7: Least squares means for feed efficiency (average total DMI: average total BW gain) for calves fed milk replacer containing 0 (●), 4 (■), or 8g (▲) resistant starch (RS). Main effect of week ($P<0.01$).

Least squares means for body temperatures and fecal scores are presented in Table 5 and Figures 8 and 9. There was no main effect of treatment ($P>0.05$) or week ($P>0.05$) on recorded daily body temperatures (Figure 8). Despite a treatment by week interaction ($P<0.05$), average body temperatures among the three treatments in our experiment were considered to be within normal ranges and consistent with other research (Hill et al., 2009). Although there was a treatment by week interaction ($P<0.05$) seen in fecal scores, all fecal scores were within normal range for healthy calves. An improvement ($P<0.05$) in fecal scores was seen as animals aged (Figure 9). Terre et al. (2006) found no significant difference in fecal consistency in calves supplemented with MOS while both Heinrichs et al. (2003) and Hill et al. (2009) observed a reduction in fecal scores in response to MOS and/or yeast supplementation in preweaned dairy calves.

Table 5: Least squares means for average body temperature, fecal score, and fecal pH for calves fed milk replacer containing 0, 4, or 8g resistant starch (RS).

	Treatment				
Item	0g	4g	8g	SEM	P-Value
Body Temperature, °C					
Entire Trial	39.47	39.47	39.47	0.59	0.95
Fecal Score¹					
Entire Trial	2.60	2.58	2.58	0.05	0.95
Fecal pH					
Entire Trial	7.67 ^a	7.44 ^{ab}	7.29 ^b	0.11	0.05

¹ 1 = normal, 2 = soft, 3 = runny, and 4 = watery

^{ab} Means with different superscripts differ (P<0.05)

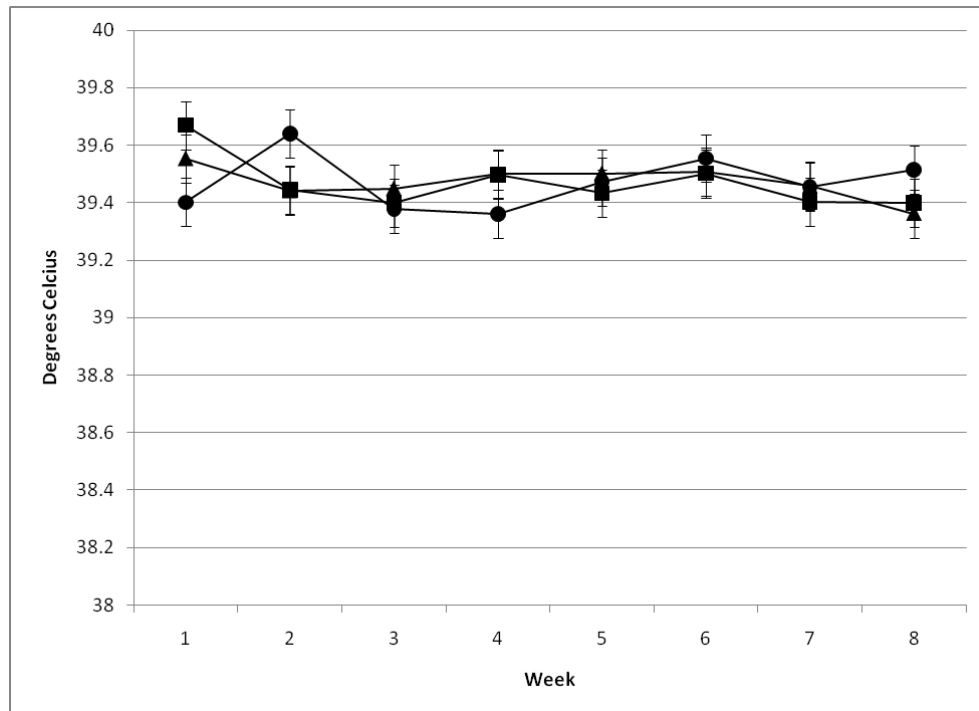


Figure 8: Least squares means for average of body temperatures for calves fed milk replacer containing 0 (●), 4 (■), or 8g (▲) resistant starch (RS). Treatment by week interaction (P<0.05).

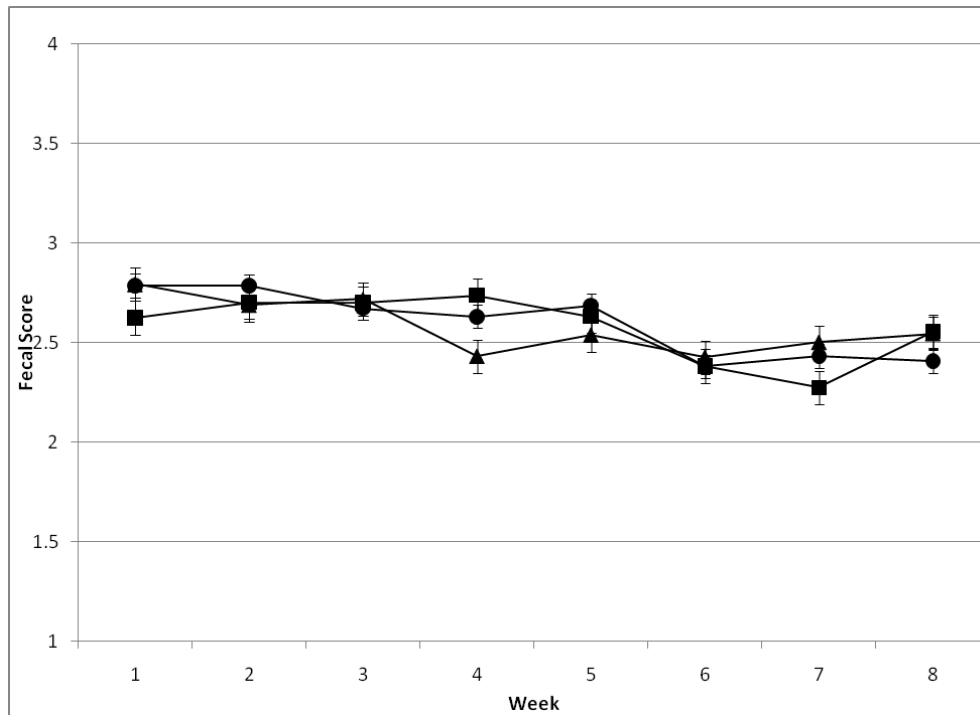


Figure 9: Least squares means for average fecal scores for calves fed milk replacer containing 0 (●), 4 (■), or 8g (▲) resistant starch (RS). Fecal Score: 1 = normal, 2 = soft, 3 = runny, and 4 = watery. Main effect of week ($P<0.05$); Treatment by week interaction ($P<0.05$).

Least squares means for fecal pH are presented in Table 5 and Figure 10. There was no treatment by week interaction ($P>0.05$) on fecal pH; however there was both a main effect of treatment ($P<0.05$) and week ($P<0.05$). Calves consuming 8g RS had a lower ($P<0.05$) fecal pH than those consuming 0g RS over the course of the experiment. However the 4g RS calves did not differ ($P>0.05$) significantly from the 0 or 8g RS groups (Table 5). At week 2 and 4, calves fed 0g RS had higher ($P<0.05$) fecal pH compared to those fed 8g RS (Figure 10). Several studies have reported a reduction in fecal pH as a result of various prebiotic supplementations in various species including RS in humans (Phillips et al., 1995), FOS and galactooligosaccharides (GOS) in infants (Knol et al., 2005), high amylose maize starch (HAMS) in rats (Le Leu et al., 2003), and FOS in horses (Berg et al., 2005). Phillips et al. (1995) reported a reduction of 0.6 pH units in humans after RS supplementation, while Berg et al. (2005) observed a reduction in

fecal pH of horses as the level of FOS supplementation increased. A reduction in fecal pH has been correlated with an increase in SCFA fermentation in the colon and may aide in preventing the proliferation of more pathogenic bacteria while allowing beneficial bacteria to flourish.

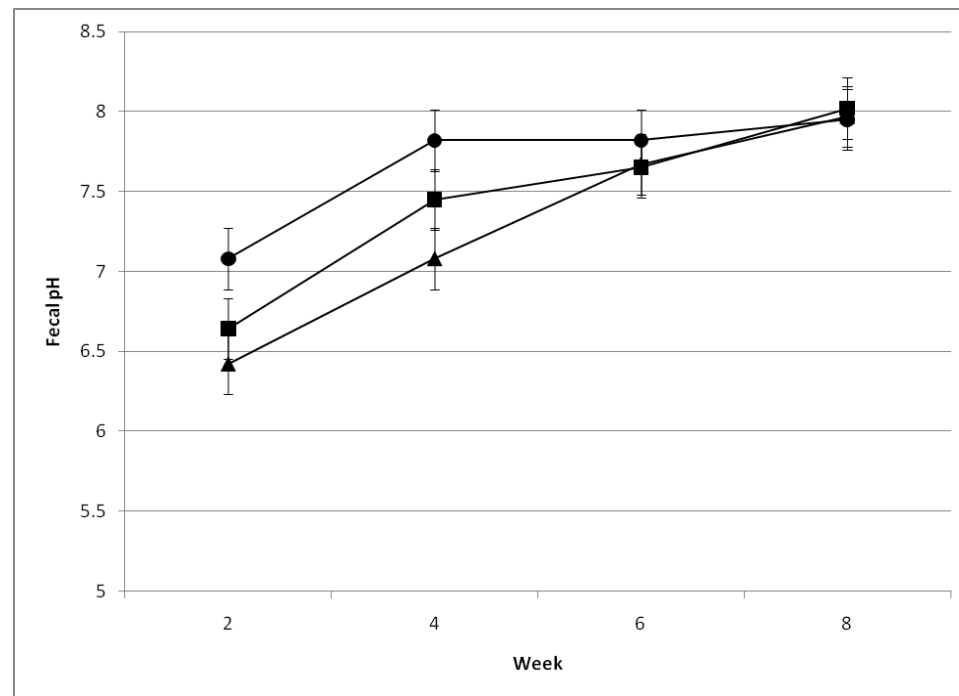


Figure 10: Least squares means for average fecal pH for calves fed milk replacer containing 0(●), 4(■), or 8g (▲) resistant starch (RS). Main effect of week ($P<0.05$) and treatment ($P<0.05$).

Least squares means for average total SCFA concentrations are located in Table 6 and Figure 11. No treatment by week interaction ($P>0.05$) or main effect of treatment ($P>0.05$) was observed in total fecal SCFA concentrations. Total fecal SCFA concentrations decreased ($P<0.01$) for all three treatments until weaning at week 6. The control calves were not significantly different ($P>0.05$) from those fed 4g RS at any point during the experiment, while at week 2 calves fed 4g RS had higher ($P<0.05$) total SCFA concentrations than those fed 8g RS (Figure 11). Research with RS supplementation in rats (Kleessen et al., 1997; Keenan et al.,

2006) and humans (Phillips et al., 1995) showed increasing levels of RS resulted in increasing levels of total SCFA concentrations while Topping et al. (1997) reported no effect of RS supplementation in pigs on total SCFA concentration in feces.

Table 6: Least squares means for average total SCFA and individual SCFA concentrations for calves fed milk replacer containing 0, 4, or 8g resistant starch (RS).

Item	Treatment			SEM	P-Value
	0g	4g	8g		
SCFA Concentrations					
Total SCFA ¹ , mmol/L	49.46	54.21	47.15	4.25	0.49
Acetate, mmol/L	32.64	34.44	33.23	2.59	0.88
Propionate, mmol/L	11.64 ^{ab}	14.08 ^a	9.38 ^b	1.20	0.03
Butyrate, mmol/L	5.18	5.68	4.54	0.64	0.45

¹Total SCFA = Acetate + Propionate + Butyrate

^{ab} Means with different superscripts differ (P<0.05)

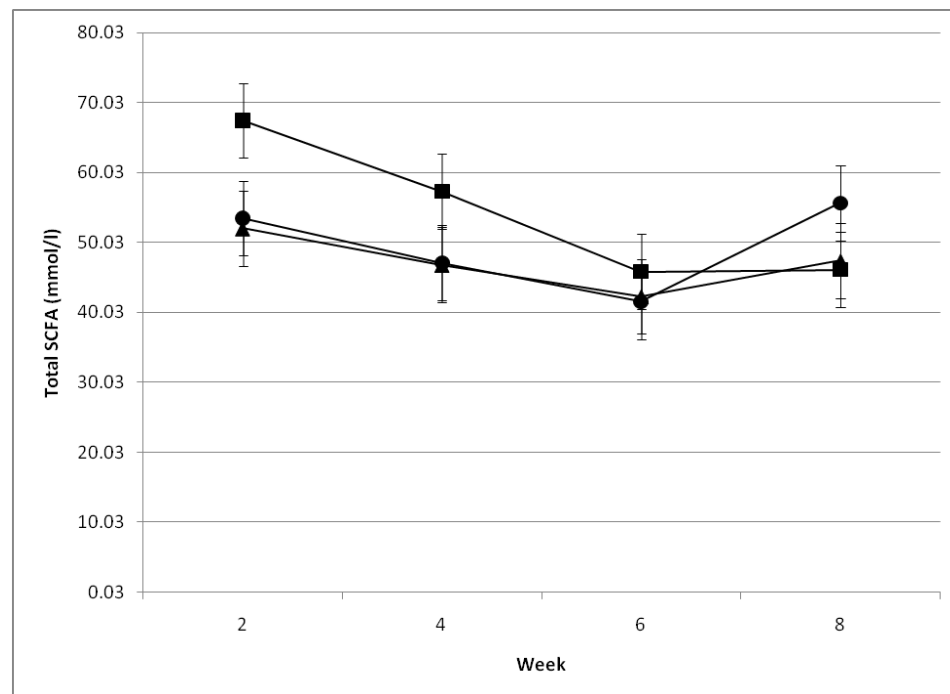


Figure 11: Least squares means for average total fecal SCFA concentrations for calves fed milk replacer containing 0(●), 4(■), or 8g (▲) resistant starch (RS). Main effect of week (P<0.01).

The major SCFAs include acetate, propionate, and butyrate are considered good markers for colonic fermentation and overall gut health (Nugent, 2005). Least squares means for average SCFA concentrations are presented in Table 6 and Figures 12, 13, and 14. In the present study there was no treatment by week interaction ($P>0.05$) or a main effect of treatment ($P>0.05$) on acetate concentrations. There was a main effect of week ($P<0.05$) on acetate concentrations (Figure 12). Acetate was the SCFA found in the greatest quantity for all treatment groups, and a decrease ($P<0.05$) was observed in all groups through weaning at week 6. Kleessen et al. (1997) and Phillips et al. (1995) reported greater concentrations of acetate in the feces of rats and humans after RS supplementation; while Topping et al. (1997) did not observe a difference in acetate concentrations in pigs fed high amylose maize starch (HAMS). For fecal propionate concentrations, a treatment by week interaction ($P<0.05$) as well as a main effect of both treatment ($P<0.01$) and week ($P<0.05$) was noted. Calves receiving 4g RS had higher ($P<0.05$) fecal propionate concentrations compared to those receiving 8g RS throughout the experiment (Table 6). At week 2 calves consuming 4g RS displayed a higher ($P<0.05$) propionate concentration than the groups consuming 0 or 8g RS. The 4g RS calves continued to have a higher ($P<0.05$) concentration of propionate in the fecal material through week 4 compared to calves fed 8g RS, while a trend ($P=0.08$) was present for calves receiving 4g RS having higher propionate concentrations than those consuming 0g RS (Figure 13). In humans, no effect of RS supplementation on fecal propionate concentrations was found after consuming 26-50g/d of Hi-maize kernels and green bananas (Phillips et al., 1995) or 17-25g/d of HAMS (Noakes et al., 1996). Kleessen et al. (1997) in rats supplemented with modified potato starch at 10% of the diet and Topping et al. (1997) in pigs supplemented with HAMS found increased propionate concentrations in fecal material. Butyrate concentrations indicated no treatment by week

interaction ($P>0.05$) or main effect of treatment ($P>0.05$). All groups had decreasing ($P<0.05$) butyrate concentrations through weaning at week 6 (Figure 14). While no main effect of RS ($P>0.05$) was observed, calves fed 4g RS had higher ($P<0.05$) butyrate concentrations than those fed 8g RS at week 2 while the control calves did not differ significantly from the other treatments. In adult subjects consuming 25% of their carbohydrate intake as HAMS (Noakes et al., 1996; Phillips et al., 1995), significantly greater butyrate concentrations were found in the fecal material when compared to the control groups while Topping et al. (1997) did not observe a difference in butyrate concentrations in pigs fed HAMS.

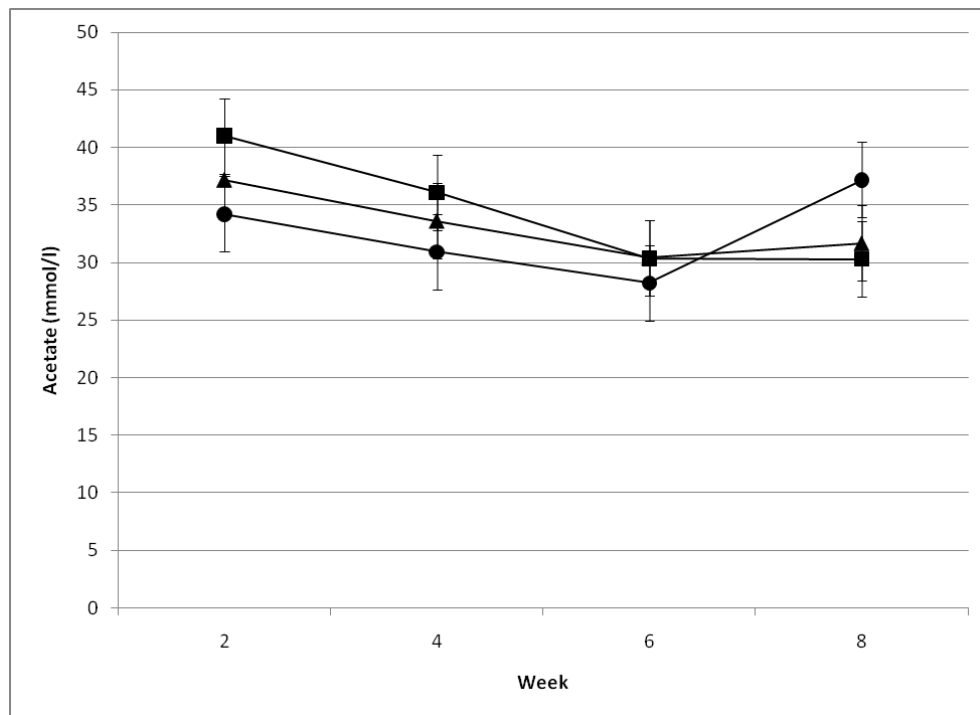


Figure 12: Least squares means for average fecal acetate concentrations for calves fed milk replacer containing 0 (●), 4 (■), or 8g (▲) resistant starch (RS). Main effect of week ($P<0.05$).

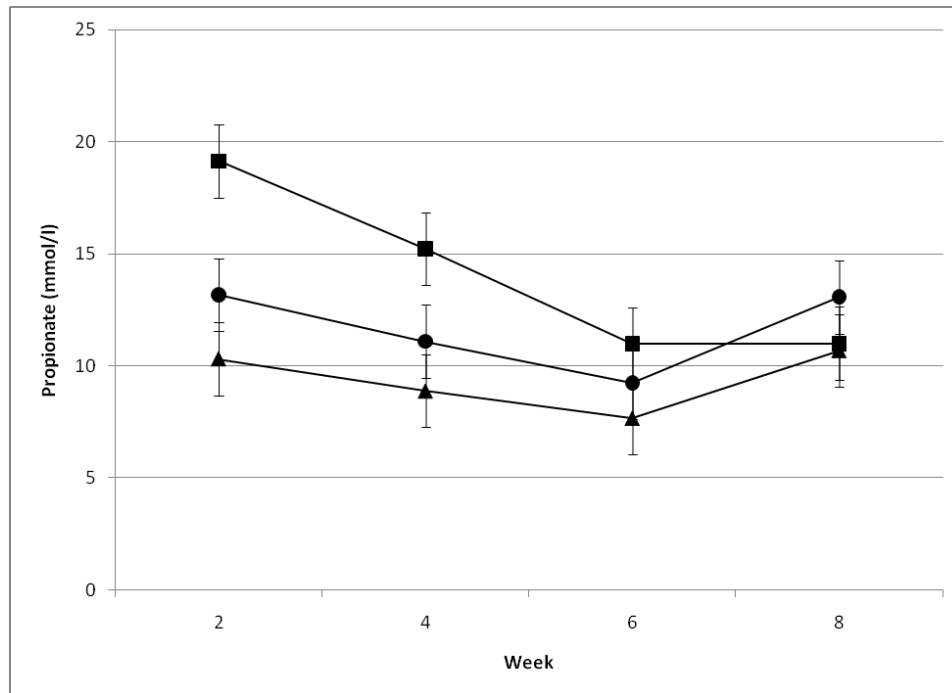


Figure 13: Least squares means for average fecal propionate concentrations for calves fed milk replacer containing 0(●), 4(■), or 8g (▲) resistant starch (RS). Treatment by week interaction ($P<0.05$); Main effect of treatment ($P<0.01$) and week ($P<0.05$).

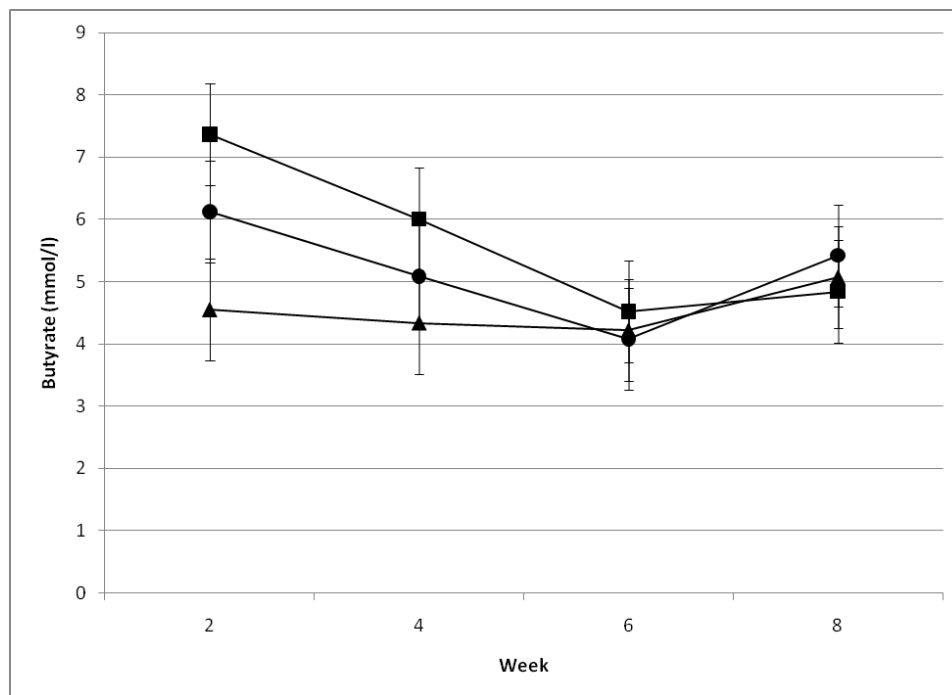


Figure 14: Least squares means for average fecal butyrate concentrations for calves fed milk replacer containing 0(●), 4(■), or 8g (▲) resistant starch (RS). Main effect of week ($P<0.05$).

Cummings et al. (1987) observed that concentrations of SCFAs in fecal material may not be the best measure of the metabolites in the feces and suggest that molar percentages of the major SCFAs in fecal material may be a better means of expressing a change. Least squares means for average SCFA molar percentages are presented in Table 7 and Figures 15, 16, and 17. There was no treatment by week interaction ($P>0.05$) on the molar percentages of acetate, propionate, or butyrate in the feces. Both main effects of treatment ($P<0.05$) and week ($P<0.05$) were observed for the molar percentages of acetate and propionate. For acetate, the main effect of treatment indicated that calves fed 8g RS had a higher ($P<0.05$) molar percentage than those fed either 0 or 4g RS (Table 7). Calves consuming 8g RS had significantly higher ($P<0.05$) molar percentages of acetate present in the feces compared to both 0 and 4g RS at weeks 2 and 4, as well as the 4g RS at week 6 (Figure 15). A trend ($P=0.07$) was observed at week 6 for calves fed 8g RS having higher molar percentages of acetate than those fed 0g RS. Knol et al. (2005) reported a higher molar percentage of acetate in infants consuming formula supplemented with FOS and galactooligosaccharides (GOS). For propionate, calves fed 8g RS had a lower ($P<0.05$) molar percentages than those fed 0 or 4g RS over the entire trial (Table 7). At weeks 2, 4, and 6, calves fed 0 or 4g RS had higher ($P<0.05$) molar percentages of propionate present in the feces compared to those fed 8g RS (Figure 16). Kleessen et al. (1997) reported higher molar percentages of propionate in rats when supplemented with RS at 10 % of daily energy intake, while Knol et al. (2005) observed lower molar percentages of propionate in infants after supplementation with FOS and GOS. Molar percentages of butyrate were not affected by treatment ($P>0.05$), week ($P>0.05$), or treatment by week interactions ($P>0.05$). However, at week 2 calves consuming 0g RS had significantly higher ($P<0.05$) molar percentages of butyrate than those consuming 8g RS, while a trend ($P=0.08$) for calves fed 4g RS having higher molar

percentages of butyrate than those fed 8g RS was observed (Figure 18). Knol et al. (2005) reported no significant effects on molar percentages of butyrate after supplementing infants with FOS and GOS while Bird et al. (2009) reported higher molar percentages of butyrate in pigs consuming HAMS.

Table 7: Least squares means for average molar percentages of acetate, propionate, and butyrate for calves fed milk replacer containing 0, 4, or 8g resistant starch (RS).

Item	Treatment			SEM	P-Value
	0g	4g	8g		
Molar Percentages of SCFA					
Acetate, mol/100 mol	66.67 ^a	65.22 ^a	70.57 ^b	0.96	<0.01
Propionate, mol/100 mol	23.24 ^a	24.64 ^a	19.97 ^b	0.74	<0.01
Butyrate, mol/100 mol	10.09	10.14	9.45	0.49	0.54

^{ab} Means with different superscripts in the same row differ (P<0.05)

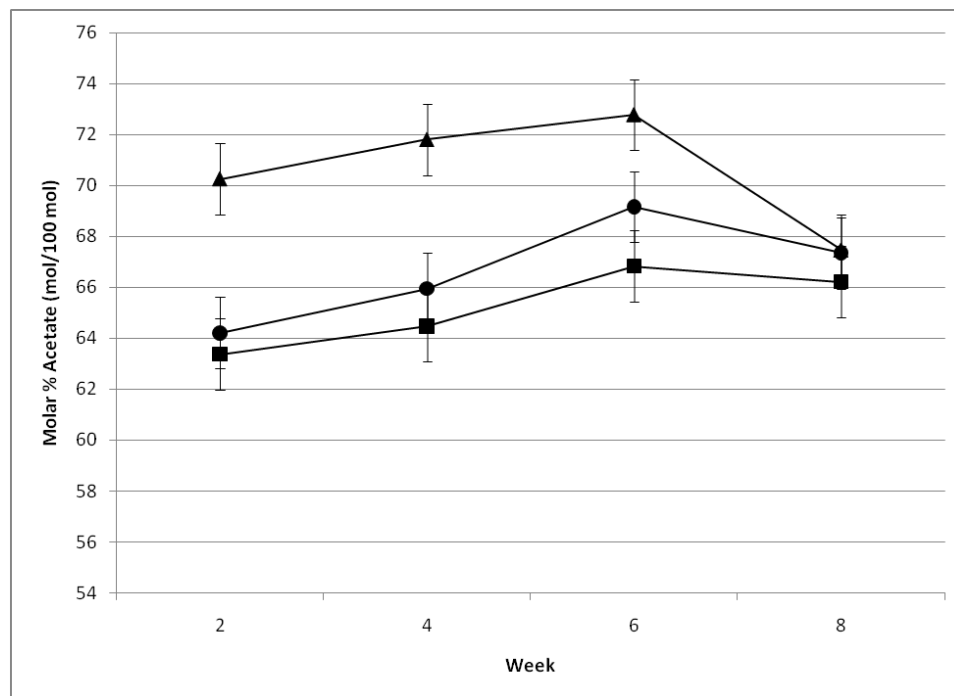


Figure 15: Least squares means for average molar percent of fecal acetate for calves fed milk replacer containing 0(●), 4(■), or 8g (▲) resistant starch (RS). Main effect of week (P<0.05) and treatment (P<0.05).

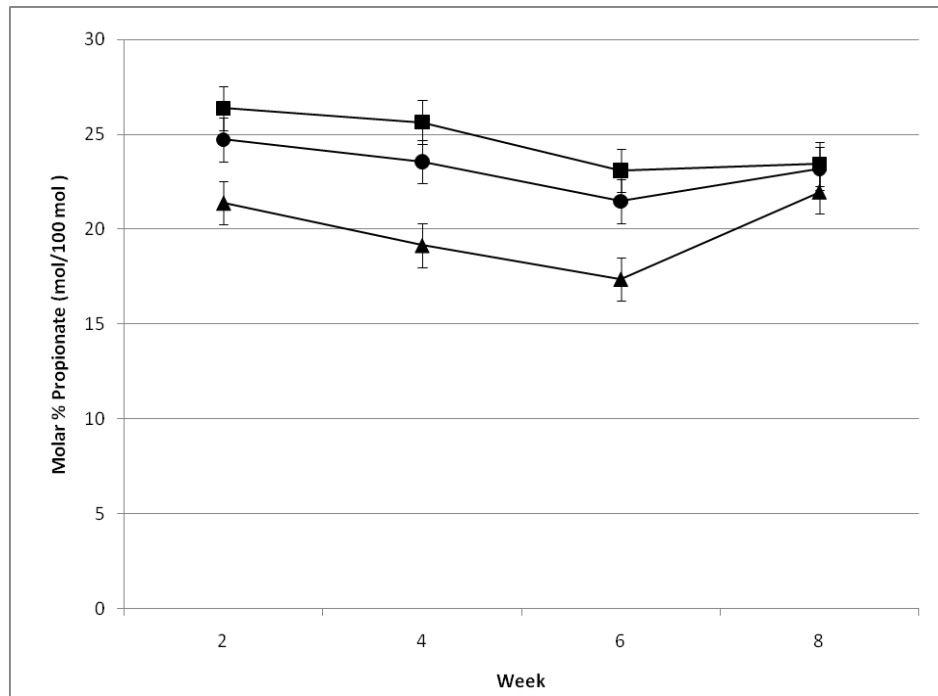


Figure 16: Least squares means for average molar percent of fecal propionate for calves fed milk replacer containing 0(●), 4(■), or 8g (▲) resistant starch (RS). Main effect of week ($P<0.05$) and treatment ($P<0.05$).

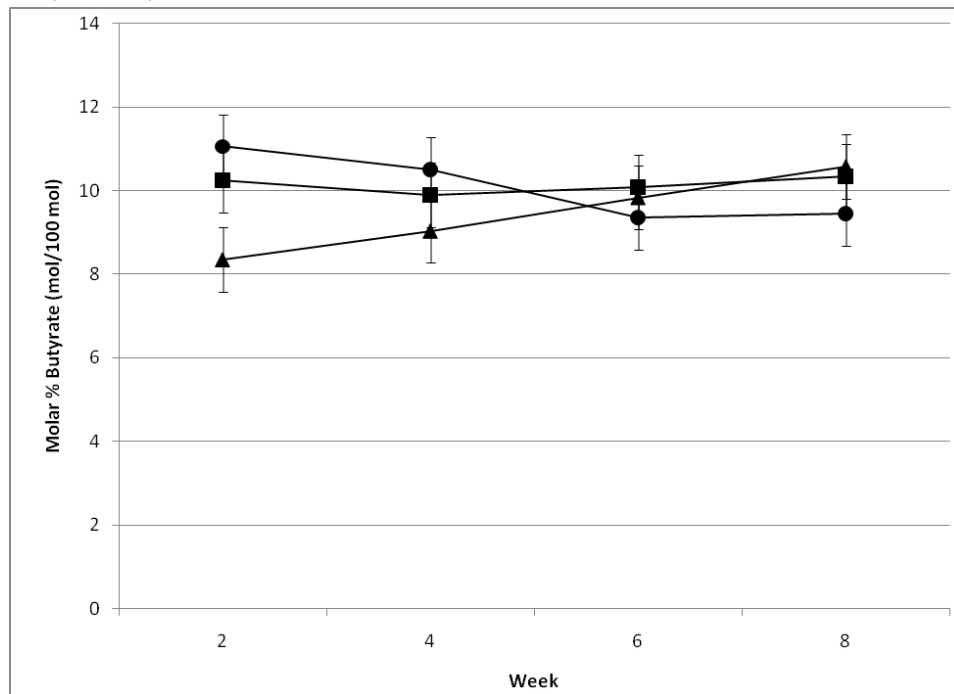


Figure 17: Least squares means for average molar percent of fecal butyrate for calves fed milk replacer containing 0(●), 4(■), or 8g (▲) resistant starch (RS).

Least squares means of average serum IgG concentrations for calves fed milk replacer containing 0, 4, or 8g RS are presented in Table 8. IgG concentrations for calves at 24hrs of age ranged from 7.12-51.65 g/L with an average concentration of 22.71 g/L. While 7.12 g/L at 24 hrs is below the recommended 10 g/L for passive transfer of immunity, all calves were healthy at the beginning of the experiment. Quigley et al. (1997) reported similar serum IgG concentrations of 18.3 g/L with a range of 2-52.2g/L.

Least squares means of average hematocrit and total protein (TP) concentrations for calves fed milk replacer containing 0, 4, or 8g RS are presented in Table 8. There was no effect of treatment ($P>0.05$) or treatment by week interaction ($P>0.05$) for TP or hematocrit. An increase over time ($P<0.05$) was observed for hematocrit values for all treatments. Hematocrit values for calves ranged from 18-45% with an average of 35.3% which is consistent with those reported by Quigley et al. (2002). Serum TP levels for calves ranged 3.04-9.46 mg/dl with an average concentration of 6.19 mg/dl. Previously reported values ranging from 5.3-5.9 mg/dl are within our observed values (Heinrichs et al., 2003; Donovan et al, 2002; Hill et al., 2008) indicating normal values for healthy calves.

Least squares means of average plasma urea nitrogen (PUN) concentrations for calves fed milk replacer containing 0, 4, or 8g RS are presented in Table 8. There was a treatment by week interaction ($P>0.05$), main effect of week ($P<0.05$), and main effect of treatment ($P<0.05$) for PUN concentrations. For all calves PUN concentrations increased ($P<0.05$) over the course of the experiment. Calves consuming 8g RS were lower ($P<0.05$) than those consuming 0 or 4g RS over the entire experiment as well as at week 8. Despite these effects, this data indicated that all values were within normal ranges and no major metabolic problems were present among any of the treatment groups throughout the course of the experiment. For our study, PUN

concentrations ranged 6.55 –25.04 mg/dl with an average concentration of 11.73 mg/dl.

Heinrichs et al. (2003) reported PUN levels of 10 ± 2.7 mg/dl in healthy calves, which is consistent with our reported values.

Table 8: Least squares means for average serum IgG, hematocrit, serum total protein, and plasma urea nitrogen for calves fed milk replacer containing 0, 4, or 8g resistant starch (RS).

Item	Treatment			SEM ¹	P-Value
	0g	4g	8g		
24 hr Serum IgG, g/L	24.29	22.15	21.68	NA	0.74
Hematocrit, %PCV	30.60	31.64	28.97	0.93	0.14
Total Protein, mg/dl	6.33	6.20	6.05	0.19	0.59
Plasma Urea Nitrogen, mg/dl	12.39	12.30	10.49	0.35	<0.01

¹SEM=Standard Error of Means

Chapter 5

Summary and Conclusion

Summary

A study was conducted to determine the effects of RS supplementation in milk replacer on health and performance of dairy calves. Forty-two female Holstein heifer calves were assigned to one of three treatments which included a control with no RS (0g), 4 g RS, or 8g RS. Calves were removed from their dams on d 0, weighed, vaccinated and placed in a hutch, measuring 2.5 m² and connected to a wire enclosure measuring 2.3 m². Navels were disinfected with a 4% iodine solution, and 3.4 liters of colostrum were fed within 3 hours of birth. Calves were fed milk replacer once daily until d 42 of age. An 18% crude protein calf starter and water were offered *ad libitum* beginning d 3 throughout the duration of the trial. Calves remained in their hutches until 56 d of age to determine immediate postweaning performance.

Feed intake, fecal scores, and body temperatures were recorded daily beginning at d 3 until d 56. Body weights were measured at birth, 7, 14, 28, 42, and 56 d of age. Withers height (WH), hip height (HH), and hip width (HW) were measured 7, 14, 28, 42, and 56 d of age. Fecal samples were collected at 14, 28, 42, and 56 d of age. Fecal samples were analyzed for pH and SCFA. Blood was collected at 24 hr of age for IgG analysis. At 14, 28, 42, and 56 d of age blood was collected for analysis of TP, PUN, and hematocrit.

Overall means for BW, HH, HW, and WH were not affected ($P>0.05$) by treatment. Overall mean average daily starter dry matter intake and total dry matter intake were not affected ($P>0.05$) by the inclusion of 4 or 8g of RS in the milk replacer. However BW, HH, HW, and WH did increase ($P<0.05$) over time which was expected with the increase ($P<0.05$) in starter DMI and total DMI. Fecal scores were not affected ($P>0.05$) by RS supplementation, however a

treatment by week interaction ($P<0.05$), as well as, a main effect of week ($P<0.05$) was observed. All fecal scores increased with age and were within a normal range for healthy calves. Body temperatures were not affected ($P>0.05$) by treatment or week. While a treatment by week interaction ($P<0.05$) was observed in body temperatures, all body temperatures were within normal range for healthy calves.

Fecal pH was affected ($P<0.05$) by treatment in which the pH was lower when 8g RS was consumed. There was no treatment effect ($P>0.05$) for total fecal SCFA concentrations, however a decrease ($P<0.05$) was observed for all treatments until weaning at week 6. There was no treatment by week interaction ($P>0.05$) or a main effect of treatment ($P>0.05$) on acetate or butyrate concentrations. There was a main effect of week ($P<0.05$), where both acetate and butyrate concentrations decreased for all treatments until week 6. There was a treatment by week interaction ($P<0.05$) as well as a main effect of both treatment ($P<0.05$) and week ($P<0.05$) for fecal propionate concentrations. The calves fed 4g RS had higher fecal propionate concentrations compared to those fed 8g over the entire trial. Calves consuming 4g RS had higher ($P<0.05$) propionate concentrations than those consuming 0 g RS at week 2 and 8g RS at weeks 2 and 4.

Main effects of treatment ($P<0.05$) and week ($P<0.05$) were observed for molar percentages of acetate and propionate. For acetate, a treatment effect was evident with calves consuming 8g RS having higher ($P<0.05$) molar percentages than those consuming 0 or 4g RS over the entire trial. At weeks 2 and 4, calves fed 8g RS had higher ($P<0.05$) molar percentage of acetate in the feces compared to those fed 0g RS. Calves consuming 8g RS also had higher ($P<0.05$) molar percentages of acetate compared to those consuming 4g RS at weeks 2, 4, and 6. For propionate, calves consuming 8g RS had a lower ($P<0.05$) proportion than calves consuming

0 or 4g RS. At weeks 2, 4 and 6, calves fed 8g RS had a lower ($P<0.05$) molar percentage of propionate present in the feces compared to those fed 0 or 4g RS.

Serum IgG concentrations were within normal ranges. Total protein and hematocrit were not affected ($P>0.05$) by treatment. Despite the effect of treatment ($P<0.05$) on PUN concentrations reported values indicated normal metabolism of calves. All values reported are consistent with previous research and indicate an overall healthy animal.

Conclusions

IgG concentrations indicated passive transfer of immunity was achieved for all calves prior to treatment. Body temperatures, TP, PUN, and hematocrit were all within normal ranges suggesting that there were no major metabolic problems in any of the treatment groups. The addition of RS had no significant effect on BW, HH, HW, WH or fecal scores in preweaned dairy calves. Fecal scores, BW, HH, HW, and WH increased throughout the duration of the trial which was expected with the corresponding increase also noted in DMI. The reduction in fecal pH along with the change of molar percentages of acetate and propionate indicates that RS may have a positive influence on bacterial fermentation and gastrointestinal health of the neonatal calf.

However, further research in this area is necessary to determine health effects in neonatal calves. Calves in all treatment groups were healthy and displayed no signs of illness throughout the experiment, therefore it is possible that the use of RS as a prebiotic may be more effective in calves challenged with disease. Differences in fecal pH and in fecal SCFA concentrations and molar percentages were seen in calves fed RS at week 2. As calves began to consume more starter grain the differences observed in these variables became smaller and non significant by weaning at week 6. RS and milk replacer were fed at a constant amount through weaning while

starter DMI gradually increased through week 8. Research to study effects of RS on hindgut fermentation in calves from birth to 3 weeks without possible confounding effects of starter DMI could help further explain the changes seen in the current study. Incorporation of RS in the milk replacer of calves did show changes in fecal SCFA at 2 and 4 weeks of age. However, incorporation of RS in milk replacer had no overall treatment effects on health and performance of neonatal dairy calves.

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Appendix A: Analysis of Short Chain Fatty Acids in Feces

Based on preparation procedures described in Grigsby et al., 1992. J. Anim. Sci. 70:1941-1949, and temperature gradient program described in Bateman et al., 2002. Prof. Anim. Sci. 18:363-367.

Reagents

1) 25% (wt/vol) metaphosphoric acid (fluka #79615) acid solution containing 2 g/L of 2-ethyl butyric acid (216.5 μ L 2-EB to 100 mL m-phos acid solution; Aldrich #10, 995-9).

2) SCFA standard

a) Add the following volumes of acids to a 100-mL volumetric flask and fill volume with dH₂O. Store in refrigerator when not in use.

MW	Acid	Volume (μ L)	Conc (g/L)	Conc (mM)
60.06	Acetic	330	3.46	57.62
74.08	Propionic	400	3.97	53.59
88.10	Isobutyric	30	0.29	3.29
88.10	Butyric	160	1.53	17.37
102.13	Isovaleric	40	0.375	3.67
102.13	n-Valeric	50	0.471	4.61

Sample and Standard Preparation

1) Centrifuge strained ruminal fluid at 30,000 x g for 20 min (this step may be skipped).

2) Mix 4 mL of rumen fluid supernatant with 1 mL of m-phosphoric acid solution containing 2-EB.

3) Allow to stand in ice bath for 30 min (this step may be skipped).

4) Centrifuge at 30,000 x g for 20 min.

5) Remove the supernatant for GC analysis.

6) To insure that standard is prepared in the same manner as the samples, treat the mixed sample from step A-2 above as a sample.

Remember to correct the dilution factor from the m-phos solution when calculating the final VFA concentrations (4mL fluid mixed with 1 mL acid provide a correction factor of 1.25).

For use on Shimadzu GC, samples should be in 2 mL autosampler vials. The optimal vials that we have used are ordered from Cole-Parmer. They are Target autosampler vials (#A98810-00).

These are a screw cap vial so you also need caps, and the septa color is important. The autosampler recognizes white as the color of the septa (#A98801-23).

Temperature Gradient Program

1) The column temperature at the beginning of the program is 115°C and is held there for 0.1 min.

2) It is then increased at a rate of 10°C/min to 150°C and held there for 0.1 min.

3) It is then further increased at a rate of 11°C/min to 170°C and held there for 1 min.

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4) The injector of the chromatograph is held at 250°C and the detector is held at 275°C.

5) Peak detection is by a flame ionization that uses a H₂/ air flame.

6) Helium is used as the carrier gas with a splitless injection at a flow of 60 mL/min.

Appendix B: Urea Nitrogen (Bun) Berthelot/Colorimetric Assay

Reagents:

- 1) Enzyme Reagent (ENZYME RGT)
- 2) Color Reagent (COLOR RGT)
- 3) Base Reagent (BASE RGT)
- 4) Standard (25 mg/dL)

Procedure:

- 1) Transfer 0.5 ml of COLOR RGT to vials labeled; unknown, control, standard, blank.
- 2) Add 0.010 ml (10 μ L) of sample to its corresponding vial.
- 3) Add 0.5 mL of ENZYME RGT to all vials, mix gently, and incubate at 37°C for five minutes. (Alternative: React for 10 minutes at room temperature 2- 26°C).
- 4) Add 2.0 mL of BASE RGT, mix and incubate at 37°C for 5 min. (Alternative: React for 10 minutes at room temperature 2- 26°C).
- 5) Set the wavelength of the photometer at 630nm and zero the photometer with the BLANK. Read and record the absorbencies of all vials and proceed to the Calculation with Example below.

Note: For a direct read-out instrument, set read out to concentration of Standard (25 mg/dL). Read unknown concentration directly.

Calculation:

Where A = absorbance, U = UNKNOWN, S = STANDARD, C = concentration:

$$A(U) \times C(S) \text{ mg/dL} = C(U) \text{ mg/dL}$$

$$A(S)$$

Appendix C: Total Protein (Biuret) Reagent Set

Reagents:

- 1) Total Protein Reagent (TP RGT)
 - a. Sodium Hydroxide 600mM
 - b. Copper Sulfate 12 mM
 - c. Sodium Potassium Tartrate 32mM
 - d. Potassium Iodide 30 mM
 - e. Non-reactive ingredients
- 2) Standard (8g/dl)

Procedure:

- 1) Label test tubes.
- 2) Pipette 1.0ml of working reagent to each tube.
- 3) Add 0.02ml (20µl) of standard and patients to appropriate tubes and mix by inversion.
- 4) Let the tubes stand at room temperature (18-25°C) for 5 minutes.
- 5) Set the spectrophotometer at 540nm and zero instrument with the reagent blank.
- 6) Read and record absorbance readings of each tube.

Calculations:

Where A = absorbance, U = UNKNOWN, S = STANDARD, C = concentration, TP=Total Protein:

$$\frac{A(U)}{A(S)} \times C(S) = TP(C) (D/DL)$$

Appendix D: MBC QT II™ Bovine Serum IgG

Procedure:

1. Allow the sample and reagent vial to warm to room temperature (approximately 20 minutes).
2. Insert the zero vial in the MBC QTII®. Align the ▼ on the vial with the ▲ on the adapter to obtain a continuous beeping and view ***** across the display. If ***** an beeping is not observed, the vial may need to be rotated left or right to initiate the zero feature. Cover the vial. When completed “0.000”, “ZERO”, and “0 Program” will be displayed.
3. Add 10 µL of sample to the reagent vial. Re-cap and mix the contents by inverting several times. Wipe off any liquid or smudges from the exterior of the vial.
4. Incubate the reagent vial containing the sample for 5 minutes at room temperature.
5. Mix the contents again by inverting several times. Insert the vial into the MBC QTII®. Align the ▼ on the vial with the ▲ on the adapter. Rotate the vial left or right to initiate measurement if necessary. Cover the vial.
6. The MBC QTII® will display the result in mg/dL. Record the value.

Limitations:

This method is linear to a Bovine IgG level of 1600 mg/dL. The MBC QTII® will indicate if a sample is out of range with the message “OVERRNG” or “UNDRNG”. Sample higher than 1600 mg/dL should be re-assayed after dilution with 0.9% saline. The results should then be multiplied by the dilution factor.

Calculations:

Diluted Concentration X Dilution Factor = Concentration

Vita

Bethany Leann Fisher was born September 2, 1985, and is the daughter of Tony and Linda Fisher of Des Arc, Arkansas, and the late Helen Ferrell of Dewitt, Arkansas. She graduated from Des Arc High School in May of 2003, and began her bachelor's degree in August of 2003 at Southern Arkansas University in Magnolia, Arkansas, as a Mulerider. It was here that she found her passion for dairy cows and in May of 2007 she graduated from Southern Arkansas University with a Bachelor of Science in Agriculture. In August of 2007 she began her graduate studies at Louisiana State University in ruminant nutritional physiology under the guidance of Dr. Bruce Jenny. In June of 2008 she took a research associate position at the LSU Dairy Science Teaching and Research Farm serving as the full-time Assistant Manager and a part-time graduate student. She will receive her Master of Science in animal and dairy sciences in August of 2011.