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Effects of probiotics and yeast culture on rumen development and growth of dairy calves

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EFFECTS OF PROBIOTICS AND YEAST CULTURE ON RUMEN DEVELOPMENT AND GROWTH OF DAIRY CALVES

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ABSTRACT

A study was conducted to determine the effects of probiotics and yeast culture on rumen development and growth of neonatal Holstein dairy calves. Forty-eight calves (heifers n=20, bulls n=28) were randomly assigned one of four dietary treatments which included calf starter containing no additive (control; **C**); calf starter containing the yeast culture *Saccharomyces cerevisiae* (yeast culture; **YC**); calf starter containing the probiotics *Bacillus lichenformis* and *Bacillus subtilis* (probiotic; **P**); and a calf starter containing both yeast culture and probiotics (yeast culture and probiotic; **YCP**). Calves were administered their treatments from day 2 to day 56 in calf starter and from day 57 to day 84 in calf grower diets. Body weights were measured at birth and weekly thereafter until day 112 of age. Additionally, wither and hip heights were measured weekly. Feed intake, water intake, and fecal scores were recorded twice daily until day 56 of age. Rumen fluid was collected on days 14, 28, 42, 56, 70, 84, and 112 for analysis of pH, VFA, and NH3 to evaluate possible differences in rumen development. Blood was collected on d 28, 42, 56, 84, and 112 for analysis of BHBA concentrations. There was a sex*treatment interaction ($P < 0.01$) for calf starter intake. Male calves receiving P consumed less than all other calves on the experiment. Females consuming calf starter containing no additive ate less than males on the same diet. Female calves consuming calf starter containing YC ate more than males consuming calf starter containing YC. There was a tendency ($P = 0.06$) for calves receiving starter containing YC to consume more than calves not fed YC. Calves consuming starter containing P drank less water than all other calves on the experiment ($P = 0.01$). There was a significant sex*time*treatment interaction for body weight gain ($P < 0.05$). Females consuming diets containing no additives and males consuming diets containing probiotics showed a decreased body weight over time when compared to calves on other treatments ($P <$

0.05). Calves consuming YC showed an increased body weight when compared to other calves at week 6 and 8 ($P < 0.05$). However, this result was not significant overall ($P > 0.1$). There were no differences among treatment groups for hip and wither height ($P > 0.1$). Calves consuming YC had higher fecal scores than those with no YC in their starter ($P < 0.05$). However, all fecal scores were well within normal ranges typically seen in healthy calves. There were no differences among treatment groups on pH, NH3, BHBA, butyrate, and propionate ($P > 0.1$). A significant sex*treatment*week interaction occurred for acetate concentrations ($P < 0.05$). Calves consuming feed containing P had an increase in acetate, with females showing a greater increase over males. Incorporating YC into starter may result in an increase in growth. However, this effect did not continue after weaning. Rumen development remains unaffected by the addition of YC and P to grain diets.

CHAPTER 1

INTRODUCTION

Replacement dairy heifers are the future of the dairy industry. In order for a dairy farmer raising his own heifers to have a successful operation, he or she must strive for these heifers to reach breeding size as quickly as possible. An obtainable goal is to have heifers reach an optimum breeding weight of 550-770 lb. at approximately 13 months of age (Smith, 2007). The sooner the heifers calve, the sooner they become profitable to the herd. Calf producers must find a cost effective method to have these heifers reach their breeding weight as soon as possible.

For a calf to grow efficiently, it must have a successful transition from liquid to solid feed. For a dairy heifer, the first solid feed offered is a starter diet. A grower diet is then fed after weaning, with a roughage source also offered at this time.

A proper solid feed is required to stimulate rumen development. A neonatal calf's digestion functions as a monogastric animal's, with the abomasum being the primary compartment for digestion. As the calf ingests solid feed, the rumen begins to take over as the primary compartment. Studies show that concentrates promote an increased rate of rumen development over roughages (Beharka et al., 1998; Heinrichs and Lesmeister, 2000).

Rumen development is primarily stimulated by dietary change and is characterized by three changes: 1) change in physical size; 2) change in wall thickness; and 3) papillae formation. Papillae are important because they add to the surface area of the rumen, allowing for greater absorption of nutrients. Absorption and metabolism of volatile fatty acids (**VFA**), primarily butyrate and propionate, initiate and stimulate development of papillae. Diet is extremely important to rumen growth and papillae development. Liquid feeds allow for minimal rumen

development, while solid feeds stimulate rumen microbes to proliferate, which produce VFA. Concentrates promote an increased rate of rumen development when compared to forages.

Due to the increased concern with antibiotics and other growth stimulants in the animal feed industry, research of other feed additives, such as direct-fed microbials (**DFM**), has increased. An interest in the effects of DFM on animal health and performance has heightened (Krehbiel et al., 2003).

A DFM is a feed supplement that contains viable, naturally-occuring microorganisms. When live microorganisms are added as a supplement or feed additive, a guarantee of those viable cells usually appears on the label. The United States Food and Drug Administration (FDA) requires feed manufacturers to use the term "direct-fed microbial" (Krehbiel et al., 2003).

Direct-fed microbials have been frequently added to milk replacer for gastrointestinal health benefits, as well as improving average daily gain, daily feed intake, and feed conversion. Use of these supplements in calves as a preventative practice has increased from 13.1% to 20% from 1996 to 2007 (USDA, 2008).

Probiotics and yeast culture have many benefits when added to a diet. They stimulate desirable microbial growth in the rumen and stabilize the rumen pH. Ruminal fermentation and end product production can be altered. Increase in nutrient flow postruminally, nutrient digestibility, and the alleviation of stress through enhanced immune response are other benefits of DFM (Yoon and Stern, 1995).

A probiotic is defined as a live microbial feed supplement that improves the intestinal microbial balance of the host animal (Cruywagen et al., 1995). Probiotics have been used as additives in milk replacer, and have been shown to increase growth performance and decrease

scour occurrence in dairy calves. Bacteria typical to the intestine (e.g., Lactobacilli) have shown an increased response in growth and health when compared to other bacteria (Abe et al., 1995).

Yeast cultures have also been shown to improve growth performance and health of calves when supplemented in the milk replacer. The culture is a yeast-fermented product that contains live and dead yeast cells, the media the cells were grown on, and the metabolic by-products produced by the yeast during fermentation (Linn and Raeth-Knight, 2006). When fed to cattle, yeast cultures have been shown to stimulate cellulolytic bacteria in the rumen, improve fiber digestion, and stabilize rumen pH (Rossi et al., 2006).

Based on the current knowledge of probiotics and yeast culture and the limited information on the supplemental use in calf starter and effects on rumen development, the objective of this study was to determine the effects of probiotics and yeast culture in calf starter on rumen development and growth parameters in neonatal dairy calves.

CHAPTER 2

REVIEW OF LITERATURE

RUMEN DEVELOPMENT

Background. A ruminant's stomach is divided into four compartments: the rumen, reticulum, omasum, and abomasum. At birth, the rumen is a rudimentary structure, with the neonatal calf behaving like a monogastric animal in terms of function and enzymatic activity. In the neonatal calf, the abomasum, or true stomach, serves as the primary digestive compartment. Proper rumen development is critical for the calf to make the transition from a preruminant animal to a fully functioning ruminant (Heinrichs and Lesmeister, 2005).

Rumen as a Rudimentary Structure. At birth, the reticulum, rumen, and omasum are present, but are underdeveloped and nonfunctional. Rumen muscularization, vascularization, volume, and papillae growth are minimal (Heinrichs and Lesmeister, 2005). Ruminal activity, including contractions, pressure, and regurgitation, is minimal to non-existent. The rumen wall is thin and slightly transparent. Rumen epithelial cells are incapable of converting butyrate to β–hydroxybutyrate, suggesting that these cells are metabolically inactive at birth (Heinrichs and Lesmeister, 2005). The undeveloped rumen appears to be affected by dietary intake and form of the diet (Beharka et al., 1998; Coverdale et al., 2004; Lesmeister and Heinrichs, 2004). Development of the rumen epithelium, muscularization, and increase in volume appear to occur independently and are each affected by different aspects of the feedstuffs (Heinrichs and Lesmeister, 2005).

Volatile Fatty Acids. The development of the rumen is primarily chemical being influenced by volatile fatty acid (**VFA**) metabolism and absorption in the rumen. These VFA are produced by naturally occurring microbes in the rumen. The major VFA produced are acetic,

propionic, butyric, and valeric acids (Davis and Drackley, 1998). These endproducts of microbial fermentation are absorbed and metabolized by the rumen epithelium. Stimulatory effects of VFA on the developing rumen are not equal. Butyrate is the most stimulatory, followed by propionate (Heinrichs and Lesmeister, 2005).

β-Hydroxybutyrate. β-hydroxybutyrate (**BHBA**) is an important metabolite used by the body as an energy source. Blood BHBA is an indicator of rumen development (Quigley, 1991). BHBA is converted from butyrate as it is oxidized by the rumen epithelial cells and passes through the rumen wall and therefore is a measurement of rumen epithelial metabolism (Lesmeister and Heinrichs, 2004). Weigand et al. (1975) reported that 26 to 33% of butyrate in the rumen is converted to BHBA. However, Lesmeister and Heinrichs (2004) found that reported blood BHBA values also included BHBA converted from butyrate and acetate in the liver.

Quigley et al. (1991) reported that blood BHBA concentrations increased when calf starter was offered from four days of age and continued to increase proportionately with calf starter intake. The increase in BHBA occurred at a slower rate after weaning at eight weeks of age.

Ruminal Ammonia . Ammonia (**NH3**) is a major protein metabolite in the rumen. It is the principle end product of microbial protein degradation and the nitrogen form required for most strains of rumen bacteria. Rumen ammonia concentration has been used as an indicator of microbe protein degradation and of non protein nitrogen utilization (Broderick and Kang, 1980).

A decrease in NH3 concentration is attributed to ruminal microbial proliferation, due to the increase of microbial use of available NH3 (Crocker et al, 1998). Beharka at al. (1998) reported a decrease in NH3 concentrations in Holstein bull calves, fed either a finely ground or

unground diet consisting of chopped hay and rolled grain. Lesmeister and Heinrichs (2004) observed a similar pattern when Holstein calves were fed texturized calf starters for 42 days. Their results indicated an increased incorporation of NH3 nitrogen into microbial protein.

Rumen pH. In establishing mature rumen fermentation, an optimal pH of 6.0 to 6.8 is required (Davis and Drackley, 1998). A pH in this optimum range must occur for the establishment and survival of a diverse and stable population of microorganisms.

Establishment of microbial populations in the rumen appears to follow a pattern with regards to substrates available and ruminal pH. During the first few weeks of life, rumen fermentation activity is low and pH is high. Lengemann and Allen (1959) found that calves reached an adult level of rumen microbial activity at 6 weeks of age when given access to solid feed.

Ruminal pH is controlled by multiple factors including relative concentration of bases, acids, and buffers (Owens et al., 1998). The primary base in the rumen is NH3, with lactate being the primary acid and bicarbonate and phosphate acting as major buffers.

Feedstuffs and Rumen Development. Rumen development and microbe proliferation is highly dependent on dietary intake level and type of feedstuffs. Ingestion of solid feeds stimulates rumen microbial growth and production of VFA, while calves receiving a liquid diet of milk or milk replacer (**MR**) have minimal development (Heinrichs and Lesmeister, 2005).

Liquid feeds, i.e. milk or MR, make up the initial diet of neonatal dairy calves. The chemical composition of liquid feeds limits rumen development (Heinrichs and Lesmeister, 2005). Numerous studies have reported minimal rumen development in calves on an exclusively liquid diet. Metabolic activity and VFA absorption is minimal (Heinrichs and Lesmeister, 2005), therefore limiting epithelial growth and muscularization. However, an increase in ruminal size

will continue proportionally with the growth of the calf, regardless of rumen development (Vazquez-Anon et al., 1993). A calf fed only a liquid diet may appear to grow normally, but the rumen will remain underdeveloped. At weaning this becomes apparent because the calf will become unhealthy and limited growth will occur due to its inability to digest grain and forages (Heinrichs and Lesmeister, 2000).

Solid feeds stimulate rumen development. However, they differ in their efficacy to do so. Solid feeds can be divided into two categories: concentrates and forages. Concentrates have an increased rate of rumen development when compared to forages (Heinrichs and Lesmeister, 2005).

Concentrate, or grain, intake increases microbial growth and production of VFA. Increased production of VFA, especially the stronger rumen acids, such as lactate, butyrate, and propionate, decrease the rumen pH (Heinrichs and Lesmeister, 2005). Grain intake influences rumen epithelial development and wall vascularization (Heinrichs and Lesmeister, 2000).

Forage intake maintains a higher ruminal pH due to larger particle size and increased fiber content and does not promote rumen epithelial growth (Heinrichs and Lesmeister, 2005). Forages are important, however, to promote rumen muscle development, to maintain healthy rumen epithelium, and to stimulate rumination and flow of saliva into the rumen (Coverdale et al., 2004)

Rumen Development. Rumen development is primarily affected by dietary change and involves two different aspects: change in physical size and change in wall thickness and papillae formation (Heinrichs and Lesmeister, 2005). Consequently, there are five factors required to cause development of the rumen. These factors are: 1) establishment of bacteria in the rumen; 2) volume of liquid in the rumen; 3) muscular action or outflow; 4) absorptive ability of the tissue;

and 5) feed availability.

The primary factor in rumen development is dry feed intake. Early consumption of dry feed is essential for promoting rumen development. Since grains have a greater influence over forages, it is important to have fresh, clean calf starter available at an early age. When a calf is first born, the rumen is sterile. However, by day one of age a large concentration of bacteria are present, most of these aerobic. As the calf consumes dry feed, the bacteria numbers and type change predominantly to anaerobes (Beharka et al., 1998).

Liquid in the rumen is important for bacterial growth and proliferation. Most of the water present in the rumen comes from free water intake, with little coming from milk or MR. Water should be offered to calves from an early age, resulting in increased body weight gain, starter intake, and reducing the occurrence of diarrhea (Heinrichs and Lesmeister, 2005).

Proper rumen development requires that feedstuffs move out of the rumen. Ruminal activity is measured by rumen contractions, rumen pressure, and regurgitation. Rumen contractions can be measured as early as 3 weeks of age and occur with the increase of dry feed intake (Heinrichs and Lesmeister, 2005).

Papillae growth is caused by the proliferation and growth of squamous epithelial cells. The presence and absorption of VFA, mainly butyrate and propionate, stimulate epithelial development. Larger papillae add to the surface area of the rumen wall and allow for greater absorption (Heinrichs and Lesmeister, 2005). Papillae growth is not universal in all areas of the rumen.

PROBIOTICS

Background. A probiotic (**P**) is defined as "a live microbial feed supplement that improves the intestinal microbial balance of the host animal" (Cruywagen et al., 1995). The term

probiotic describes viable microbial cultures, culture extracts, enzyme preparations, or various combinations of these. In 1908, Metchnikoff first proposed that consuming *Lactobacillus* species was desirable and prevented diseases caused by enteropathogens. The study of bacterial DFM increased after antibiotic use increased around World War II. These antibiotics destroyed naturally occurring intestinal bacteria, resulting in diarrhea. As a result, probiotic therapy interest increased. Studies involving the effects of probiotics on health and performance of ruminants have only occurred recently (Krehbiel et al., 2003).

Probiotics have been shown to have many functions, including protecting young animals against enteropathic disorders and increasing feed conversion efficiency and weight gain in growing animals (Windschitl et al., 1991). It appears that for a probiotic to have effect there must be a symbiotic relationship between the host and probiotic in terms of gastrointestinal tract environment of the host animal, conditions for growth, reproduction, or lyses of the probiotic, target of action, and the effectiveness of the probiotic. This symbiosis is particularly important in the case of functioning ruminants where the effects of P are thought to be mainly mediated by their effects on the rumen microbes (Van Eys and Den Hartog, 2003). It has been shown that certain probiotics have beneficial effects in the rumen, including the prevention of rumen acidosis (Ghorbani et al., 2002). Nocek et al. (2002) reported a reduced risk of acidosis in cannulated dairy cows in early lactation fed lactate-producing bacteria (*Lactobacillus* and *Enterococcus*) once daily for 21 days in situ. These bacteria caused the rumen microflora to adapt to the presence of lactate within the rumen.

Commonly used probiotics include, but are not limited to, *Lactobacilli*, *Streptococci*, *Entercocci*, *Bifidobacteria*, and *Propionibacteria* (Walker, 2007). Most of these have been shown to be the most active in the lower gut of a ruminant animal.

Probiotics in Calf Feeding Systems. The addition of probiotics in feeding systems has been shown to improve average daily gain (**ADG**), daily feed intake, and feed conversion. Abe et al. (1995) administered *Bifidobacterium pseudolongum* or *Lactobacillus acidophilus* in MR to neonatal calves from 7 days to 35 days of age. They reported that both probiotics tested improved body weight gain and feed intake over MR with no additive, with neither having a significant benefit over the other. In addition, feed conversion for treatment calves was superior to that of the control group. However, Windschitl et al. (1991) observed that the addition of *L. acidophilus*, *Aspergillus oryzae*, and *Bacillus subtilis* to a grain mix in Holstein calves 4 to 7 months of age had no significant effect on body measurement gains and feed efficiency.

Probiotics have been used to decrease diarrhea occurrence in many species. Timmerman et al. (2005) conducted an experiment comparing the difference between multi-species probiotics (**MSPB**) and calf-species probiotics (**CSPB**) in MR and found that CSPB reduced the incidence of diarrhea in veal calves. Abe et al. (1995), as previously discussed, administered *B. pseudolongum* and *L. acidophilus* to Holstein calves and observed a decrease in the occurrence of diarrhea. Taras et al. (2006) incorporated *Enterococcus faecium* into prestarter feed (15 to 28 days of age) and to starter feed (29 to 56 days of age) for piglets and observed a decrease in the actual percentage of piglets with post-weaning diarrhea. These findings may indicate that P may increase the resistance of pathogenic bacteria that causes diarrhea.

Cruywagen et al. (1995) found that average daily gain (ADG) was increased in calves receiving *L. acidophilus* in milk replacer. Calves not receiving the treatment lost 4% of initial body weight during the first 2 weeks of the study, while calves receiving the *L. acidophilus* treatment maintained their initial body weight. There was no difference between groups for

occurrence of diarrhea. This study concluded that there may be a beneficial effect of adding probiotics to milk replacer in the first 2 weeks of life.

Higginbotham and Bath (1993) performed two trials on *Lactobacillus acidophilus* in a combination of milk replacer and waste milk. In the first trial, Holstein calves were fed either nonviable *L. acidophilus* in milk or an untreated milk control for nine weeks. No differences were observed in average daily gain and fecal scores. In the second trial, viable and nonviable *L. acidophilus* additives were compared for 5 weeks. Starter intake was greater in the calves fed the nonviable additive. No significant differences were observed in average daily gain, fecal score, and in fecal bacterial counts.

YEAST CULTURE

Background. A yeast culture (**YC**) is a yeast-fermented feed additive that contains both live and dead yeast cells, the culture media the yeast cells were grown on, and the metabolic by-products produced by the yeast cells during fermentation (Linn and Raeth-Knight, 2006). In the dairy industry, these products were first used in cow rations to increase dry matter intake during the transition period or periods of stress (Garrett, 2000). Cellulolytic bacteria in the rumen are stimulated by YC. Fiber digestion in calves and cows is improved by adding YC to the diet. Yeast also provides growth factors, such as malate and vitamins, which stimulate lactate utilizing bacteria, which helps stabilize rumen pH preventing risk of acidosis (Rossi et al, 2006). Yeast does not grow in rumen fluid but retains metabolic activity and viability (Newbold et al., 1996).

The most common YC used in ruminant diets is *Saccharomyces cerevisiae*. *Aspergillus oryzae* is a fungal DFM, but is commonly classified under yeast DFM.

Yeast Culture in Calf Feeding Systems. Limited research has been conducted on the effects of adding YC to calf diets. An increase in body weight gain and feed efficiency in calves

has been seen when YC is added to the diet (Linn and Raeth-Knight, 2006). Results indicating calf health and immune status have been contradicting.

Martin and Nisbet (1990) found that *A. oryzae* increased NH3 production by more than 20% in an in vitro fermentation experiment with rumen fluid from Angus steers fed a concentrate diet. Newbold et al. (1996) also found that NH3 concentrations increased when sheep were fed *S. cerevisiae*. However, Dawson et al. (1990) reported that the addition of *S. cerevisiae* to a fescue hay based diet fed to steers had no effect on NH3 concentrations. Studies have indicated that YC stimulated NH3 production by the mixed ruminal population, suggesting that YC may enhance proteolysis. This increase in NH3 production may be due to the YC providing additional nutrients to the ruminal microorganisms or possibly by endogenous proteolytic activity of the YC (Arambel et al., 1987; Frumholtz et al., 1989; Martin and Nisbet, 1990).

Quigley et al. (1992) added *S. cerevisiae* to calf starter and evaluated blood and ruminal VFA and growth on weaned calves during weeks 12 through 14 of age. They found that mean body weight and ADG were unaffected by treatment. Calves fed the yeast had increased ruminal acetate and butyrate and decreased propionate when compared to the control diet.

Lesmeister et al. (2004) fed *S. cerevisiae* in calf starter to Holstein calves from 2 to 42 days of age. Average daily gain and DMI was higher for the treatment group. There were no treatment effects on rumen VFA and plasma BHBA. In a similar experiment, Holstein calves were fed a grain diet containing *S. cerevisiae* for 2 to 10 days of age. Results showed no differences in intake, ADG, and BHBA, but did indicate a significant decrease in fecal score (Magalhaes et al., 2008).

Ruppert et al. (1998) evaluated a combination of the yeast, *S. cerevisiae*, and the probiotics, *S. faecium* and *L. acidophilus* added to whole milk fed to Holstein calves from birth

through 6 weeks of age. Calves fed the milk treated with these DFM had an increase in ADG and feed intake and lower fecal scores. However, Quigley et al. (1992) studied effects of yeast culture in neonatal calves by adding *S. cerevisiae* to milk replacer from 3 to 42 days of age and found no significant effects on intake or on rate and efficiency of gain in these calves.

In summary, results of the addition of DFM to calf diets have been inconsistent. Some studies indicated an increase in growth performance and intake (Cruywagen et al., 1995; Abe et al., 1995). However, others show no difference (Higginbotham and Bath, 1993; Magalhaes et al., 2008). Results regarding improved fecal score and gut health have generally been positive (Ruppert et al., 1998; Timmerman et al., 2005) and may indicate that DFM may increase the resistance of pathogenic bacteria that cause diarrhea (Taras et al., 2006). Though limited research exists on rumen development, the addition of DFM to calf feeding systems generally results in an increase in rumen development measurements, such as VFA and β-hydroxybutyrate (Quigley et al., 1992).

CHAPTER 3

MATERIALS AND METHODS

ANIMALS AND DIETARY TREATMENTS

Forty-eight Holstein calves (heifers n=20, bulls n=28) were utilized in a sixteen week experiment to determine the effects of dietary inclusion of probiotics and yeast culture on growth and rumen development. All calves were born at the LSU Agriculture Center Research and Teaching Farm, Baton Rouge, LA, between, August, 2007 and January, 2008. All calves were housed at the LSU AgCenter Dairy Farm 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 separated from their dams at birth, weighed, and individually housed in 2.5-m^2 calf hutches with a 2.8-m^2 wire enclosure on rock bedding until d 56. Calves received 4 quarts of colostrum from their dams and were orally vaccinated against Rotavirus and Coronavirus (Calf Guard, Pfizer Animal Health, Lenexa, KS).

Day 2 and 3 of life, calves received transition milk from their dams in bottles. On d 4 of life, calves were offered MR containing decoquinate (20% protein, 20% fat; Nutra Blend LLC, Neosho, MO) at 10% of their birth weight and bucket trained. Refusal of MR, if any, was weighed and discarded. Calves were then randomly assigned to one of four dietary treatments in blocks of four according to birth date and sex as follows: control calves (**C**) receiving 0 P or 0 YC; calves receiving a minimum total CFU count of 3,20E+09 per gram of *Bacillus lichenformis* and *Bacillus subtilis* at a dose of 400 g/ton of feed (**P**) (BioPlus 2B, Chr. Hansen Biosystems, Denmark); calves receiving 2% of the supplemental yeast culture, *Saccaromyces* (**YC**) as a

percentage of feed as fed (Diamond V XP Yeast Culture, Diamond V Mills, Inc., Cedar Rapids, IA); and calves receiving P + YC at the above concentrations (**YCP**).

Calves were offered MR once daily at 10% of birth weight at AM feedings from d 4 until abrupt weaning at d 42. Calves were fed their respective treatments in an 18% crude protein **(CP)** calf starter until eight weeks of age at *ad libitum* levels. Starter (Table 1) was offered at 0630 and 1600 hours, with starter intake recorded for each calf. Calves were initially offered 227 g of starter, and remaining feed was weighed at each delivery time. Starter increased at 227 g increments when calves refused less than 36 g of feed. Water was offered *ad libitum* beginning on d 4. Calves were initially offered 3.859 kg of water, and remaining water was weighed at each delivery time. Additional water was offered (3.859 kg) when the calf consumed all water.

On d 57, calves were moved to pens with access to ryegrass pasture and free choice grass hay. Calves were offered a grower diet (Table 2) containing their respective treatments at a level of 2270 g/ calf/ day. Calves were offered water and hay at *ad libitum* levels. At 12 wk, calves were removed from treatment and placed on a control grower until the end of the experiment at 16 wk.

SAMPLE COLLECTION

Twice daily at feeding times, calves were observed and fecal scores recorded according to Larson et al. (1977). Scoring was as follows: for fecal fluidity, 1 = normal, 2 = soft, 3 = runny, and 4 = watery. Body weights were recorded beginning at birth and again at 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, and 112 d of age. Wither height and hip height were measured at 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, and 112 d of age. of age.

Rumen fluid was collected via stomach tube for analysis of VFA and NH3 at 14, 28, 42, and 56 d of age 4 hr post-feeding and 70, 84, and 112 d of age pre-feeding. Rumen fluid was

Table 1. Calf Starter Composition

Ingredients, % as Fed	C	YC	P	YCP
Rolled Corn	35.0	33.25	35.0	33.25
Kentwood Custom Heifer-R[1]	1.5	1.5	1.5	1.5
Pro-Lak	2.5	2.5	2.5	2.5
Stock Pellets 16%	10.0	10.0	10.0	10.0
Country Acres H&M	10.0	10.0	10.0	10.0
Rumensin/Vitamin E Premix[2]	1.0	1.0	----	1.0
Cargill Pellet Milk +	2.5	2.5	2.5	2.5
Dried Distillers Grain	4.0	4.0	4.0	4.0
Soybean Meal 48	3.5	3.25	3.5	3.25
Sweet Stuff	7.5	7.5	7.5	7.5
Protein Pellets (SBM)	10.0	10.0	10.0	10.0
Crimped Oats	10.0	10.0	10.0	10.0
Molasses	2.5	2.5	2.5	2.5
Rumensin/Vitamin E/Probiotic Premix[3]	----	----	1.0	----
Yeast Culture[4]	----	2.0	----	2.0

[1]Kentwood Custom Heifer-R contains Monensin 2,400 g/ton, Calcium(Min) 15.00%, Calcium(Max) 18.00%, Phosphorus(Min) 5.75%, Salt(Min) 18.00%, Salt(Max) 21.00%, Magnesium(Min) 2.60%, Potassium(Min) 0.90%, Sulfur(Min) 1.00%, Cobalt(Min) 25 ppm, Copper(Min) 800 ppm, Iodine(Min) 80 ppm, Manganese(Min) 2,700 ppm, Selenium(Min) 20 ppm, Zinc(Min) 2,750 ppm, Vitamin A(Min) 200,000 IU/lb, Vitamin D-3(Min) 45,000 IU/lb, Vitamin E(Min) 1,000 IU/lb

[2]Rumensin/Vitamin E Premix contained 94.5% dried distiller's grain, 0.5% Rumensin, and 5% Vitamin E

[3]Rumensin/Vitamin E/Probiotic Premix contained 89.5% dried distillers grain, 0.5% Rumensin, 5% BioPlus 2B, Chris Hansen Biosystems, and 5% Vitamin E

[4]Diamond V XP Yeast Culture, Diamond V Mills, Inc.

Table 2. Calf Grower Composition

Ingredients, % as Fed	C	YC	P	YCP
Rolled Corn	37.5	35.8	37.5	35.7
Kentwood Custom Heifer-R[1]	2.0	2.0	2.0	2.0
Dried Distillers Grain	10.0	10.0	10.0	4.0
Soybean Meal 48	15.0	14.8	15.0	14.8
Whole Cottonseed	5.0	5.0	5.0	5.0
Cottonseed Hulls	2.5	2.5	2.5	2.5
By-Product Mix	25.0	25.0	25.0	25.0
Molasses	2.5	2.5	2.5	2.5
Rumensin/Ca Carbonate Premix[2]	0.5	0.5	----	----
Rumensin/Ca Carb/Probiotic Premix[3]	----	----	0.55	0.55
Yeast Culture[4]	----	2.0	----	2.0

[1]Kentwood Custom Heifer-R contains Monensin 2,400 g/ton, Calcium(Min) 15.00%, Calcium(Max) 18.00%, Phosphorus(Min) 5.75%, Salt(Min) 18.00%, Salt(Max) 21.00%, Magnesium(Min) 2.60%, Potassium(Min) 0.90%, Sulfur(Min) 1.00%, Cobalt(Min) 25 ppm, Copper(Min) 800 ppm, Iodine(Min) 80 ppm, Manganese(Min) 2,700 ppm, Selenium(Min) 20 ppm, Zinc(Min) 2,750 ppm, Vitamin A(Min) 200,000 IU/lb, Vitamin D-3(Min) 45,000 IU/lb, Vitamin E(Min) 1,000 IU/lb

[2]Rumensin/Ca Carbonate Premix contained 94.5% dried distiller's grain, 0.5% Rumensin, and 5% Vitamin E

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[4]Diamond V XP Yeast Culture, Diamond V Mills, Inc.

analyzed for pH immediately, after which 1 mL of phosphoric acid (20% w/v) was added. Rumen fluid was stored frozen (-20˚ C) protected from light until analysis.

At 28, 42, and 56 d of age post-feeding and 84 and 112 d of age pre-feeding, blood was collected via jugular venipuncture for analysis of β-Hydroxybutyrate (**BHBA**). Blood collected for BHBA analysis was collected in 10 mL collection tubes containing sodium heparin, centrifuged for twenty minutes at 600 x g, and plasma separated and stored frozen (-20˚C) protected from light until analysis.

ANALYTICAL PROCEDURES

β-Hydroxybutyrate. Plasma was analyzed for BHBA using commercial spectrophotometric kits (β-Hydroxybutyrate Liquicolor® Kit; Stanbio Laboratory, Boerne, TX) (Appendix A).

Ammonia-Nitrogen. Before NH_4^+ analysis, acidified ruminal fluid was thawed at room temperature and clarified by centrifuging at 30,000 x g for 20 min. The clarified supernatants were then decanted and analyzed for NH_4^+ using a modified phenol-hypochlorite reaction adapted from Broderick and Kang (1980) (Appendix B).

Total Volatile Fatty Acids. A 4 mL sample of ruminal fliud was mixed with 1 mL of 25% (wt/wt) meta-phosphoric acid containing 10 g/L 2-ethylbutyric acid, an internal standard for VFA quantification. The mixture of ruminal fluid and meta-phosphoric acid was then centrifuged at 30,000 x g for 25 min. Concentrations of individual VFA were measured by 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 VFA analysis was adapted from Grigsby et al. (1992) and Bateman et al. (2002), respectively (Appendix C).

STATISTICAL METHODS AND CALCULATIONS

Variables measured daily were reduced to weekly means prior to analysis. All dependent variables were analyzed using the mixed procedure of SAS (Littell et al., 1998). For all variables except body weight at birth, 42 d, 56 d, 84 d, and 112 d the model included YC, P, sex, week, and their two- , three-, and four-ways interactions as fixed effects. Weekly averages were analyzed as repeated measures using a first-order auto regressive covariance structure for all variables. Block within sex and YC by P by block within sex were included into the model as random terms. Block within sex by YC by P was the subject of the repeated statement. The covariance structure was selected by choosing the best fitting model according to the Akaike Information Criterion. For body weight, hip height, and wither height response variables measured at birth were included into the model as a covariate. Weight at birth, 42 d, 56 d, 84 d, and 112 d were analyzed including in the model YC, P, sex and their two- and three-ways interaction as fixed effects. Block within sex was included into the model as random effect. Values reported are least square means. Significance was declared at $P \leq 0.05$, and a trend was reported if $0.05 < P \leq 0.10$.

CHAPTER 4

RESULTS AND DISCUSSION

PERFORMANCE DATA

Least squares means for average daily starter intake for male and female calves fed C, YC, P, or YCP are presented in Figure 1. Least squares means for overall average daily starter intake for calves fed C, YC, P, or YCP are presented in Table 3. Overall mean of average daily starter intake was not significantly affected ($P > 0.1$) by the addition of YC or P in the feedstuffs. Calves responded differently to the various treatments depending on sex ($P < 0.025$). Male calves receiving P consumed less starter. Females consuming calf starter containing no additive ate less than males on the same diet. Female calves consuming calf starter containing YC ate more than males consuming calf starter containing YC ($P < 0.05$). Because YC and P cannot be differentiated by animals based on gender, these results cannot be explained with this data. Overall means for starter intake are presented in Table 3. Calves receiving calf starter containing YC tended to consume more than other calves ($P = 0.068$). However, Quigley et al. (1992) found no significant effect of YC on intake of starter. As expected, there was a significant week effect ($P < 0.0001$). As calves aged, starter intake increased among all treatments.

Least squares means for water intake are presented in Table 3. As expected, water intake increased ($P < 0.0001$) with age regardless of treatment. Calves consuming starter containing P drank less overall than other treatment groups ($P = 0.0179$).

Least squares means for body weight of male and female calves fed C, YC, P, or YCP are presented in Figure 2. Least squares means for weekly body weight for calves fed C, YC, P, or YCP are presented in Figure 3. Least squares means for body weight of calves at 0 d, 42 d, 84

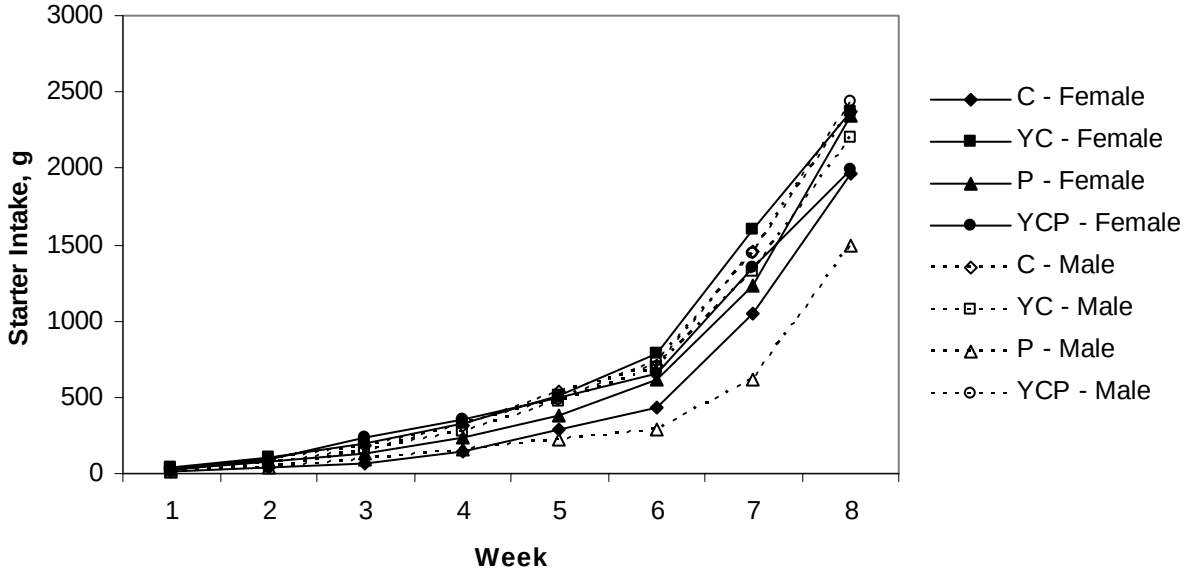

Figure 1. Weekly least squares means of daily calf starter intake for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a main effect of time ($P < 0.0001$) and a trt*sex interaction ($P < 0.025$).
SEM = 131.55

Table 3. Least squares means of average daily starter intake and water intake for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP) through day 56 of age.

	Treatment					P Value		
	C	YC	P	YCP	SEM[1]	YC	P	YC*P
Starter (g/d)	623.9	695.05	483.32	677.45	69.10	0.06	0.23	0.40
Water (g/d)	4.21	4.15	2.96	3.54	0.47	0.49	0.01	0.40

SEM[1] = Standard Error of the Mean

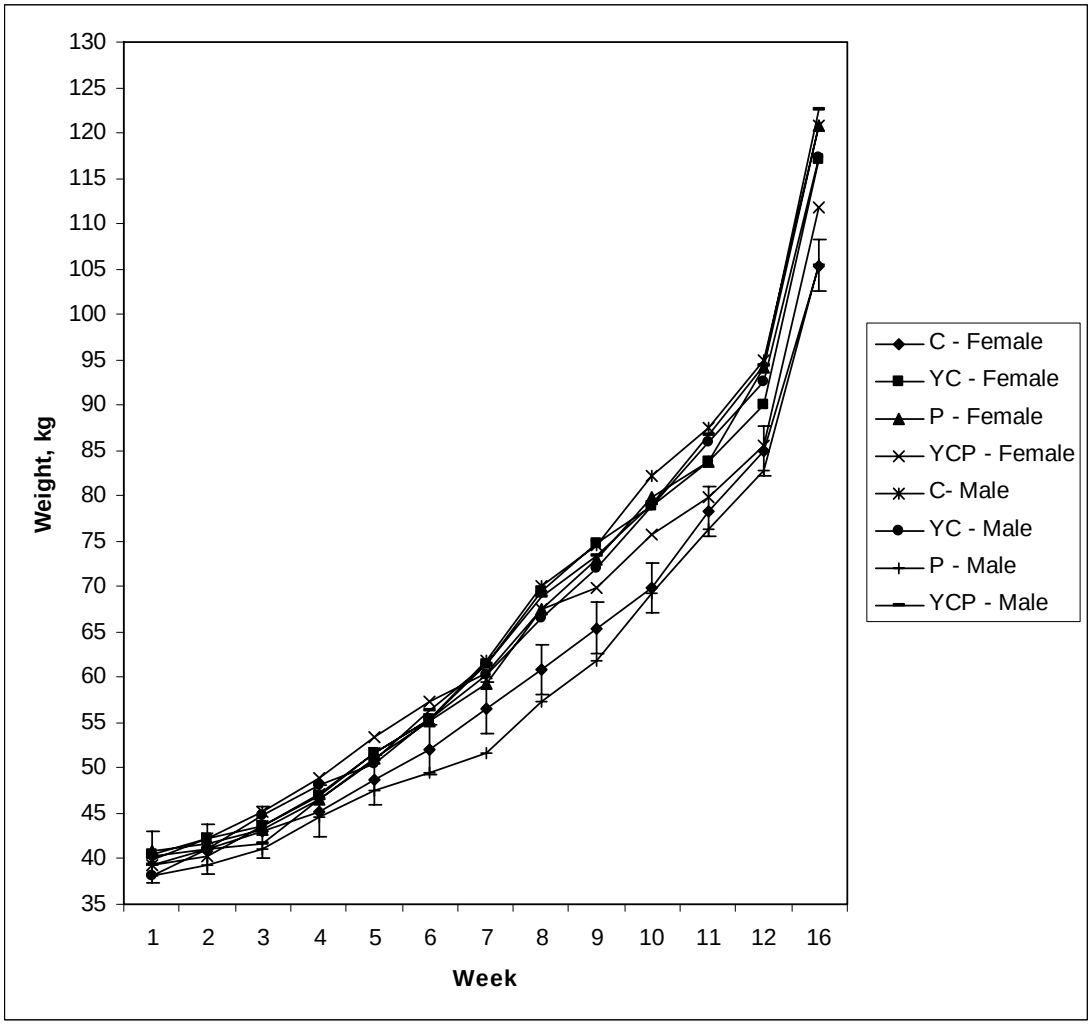

Figure 2. Least squares means for average body weight of male and female calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a main effect of time ($P < 0.05$). SEM = 2.8012

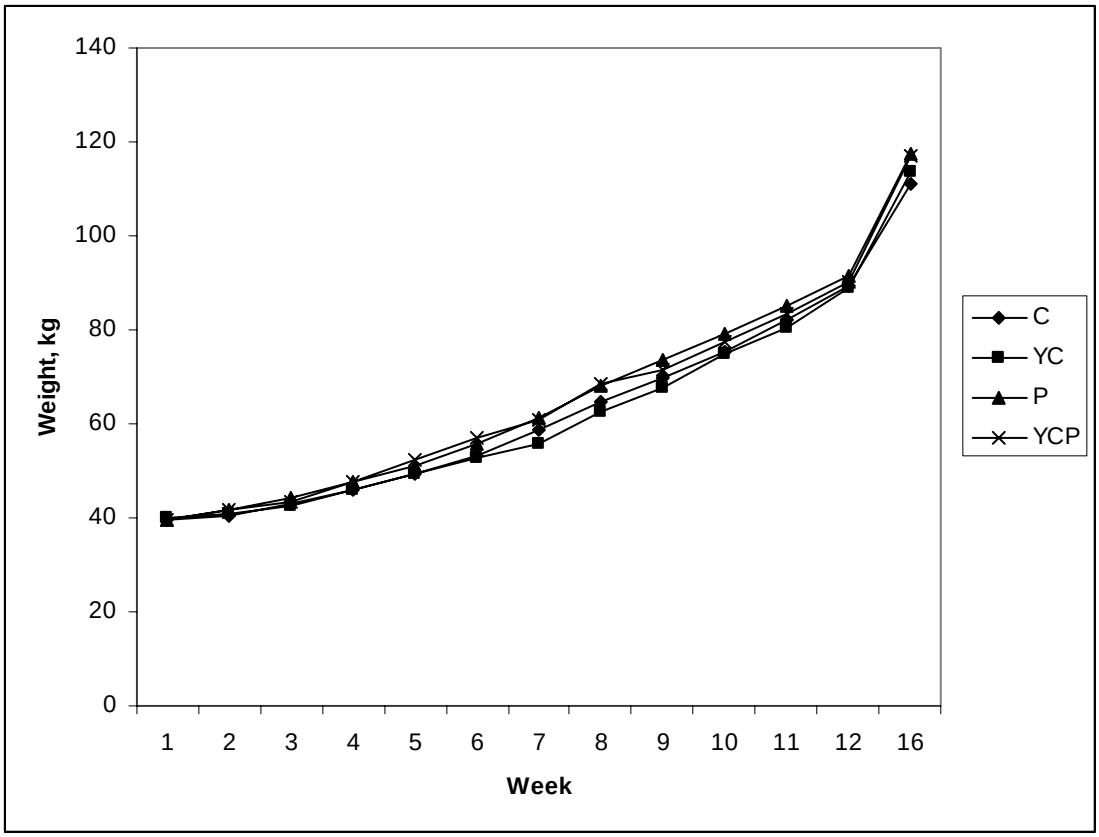

Figure 3. Least squares means for average body weight of calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a main effect of time ($P < 0.05$). SEM = 2.1404

Table 4. Least squares means for average body weight (kg) of calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP) at d 0, 42, 56, 84, and 112 of age. There was a main effect of time ($P < 0.05$).

	Treatment					P Value		
Day	C	YC	P	YCP	SEM[1]	YC	P	YC*P
Birth, d 0	36.22	38.03	38.19	37.22	1.75	0.78	0.71	0.38
Weaning, d 42	53.82	55.39	52.29	56.89	1.43	0.02	0.99	0.26
Remove from hutch, d 56	65.49	67.96	62.42	68.23	1.97	0.04	0.47	0.40
End of trt, d84	89.94	91.25	88.50	89.95	3.35	0.56	0.56	0.97
End of trial, d 112	111.27	117.05	113.05	117.29	3.79	0.16	0.77	0.83

SEM[1] = Standard Error of the Mean

d, and 112 d are presented in Table 4. Over time, calves responded differently to the various treatments depending on sex. Females consuming diets containing no additives and males consuming diets containing probiotics weighed less over time when compared to calves on other treatments ($P < 0.05$). This effect on body weight was related to starter intake. However, since P cannot differentiate gender, this result cannot be explained with this data. Calves consuming YC showed higher body weights at d 42 and d 56 when compared to calves not consuming YC ($P < 0.05$), but the effect of YC was not significant overall ($P > 0.1$). As expected, there was a significant week effect on body weight ($P < 0.0001$). In general, male calves typically have higher birth weights and greater weight gain than female calves, so a sex effect was expected. Martin et al. (1962) found relatively large differences in weight gain between sexes when evaluating factors related to weight gain in dairy calves. Quigley et al. (1992) found no significant effects of YC on ADG or intake of starter. Higginbotham and Bath (1992) reported no significant effects of P on ADG when added to waste milk. However, Lesmeister et al. (2004) reported improvement in average daily gain when 2% supplemental YC was added to a calf starter diet.

Least squares means for wither and hip height of calves fed C, YC, P, or YCP are presented in Table 5 and in Figures 4 and 5, respectively. No treatment effect was observed, but, as expected, there was a significant effect of time for wither and hip height for all treatments ($P < 0.0001$). In contrast to the current experiment, Lesmeister et al. (2004) reported increased hip height when 2% supplemental YC was added to a calf starter diet.

Least squares means for fecal scores for calves fed C, YC, P, or YCP are presented in Table 5. Calves consuming YC had higher fecal scores than those with no YC in their starter (P

Table 5. Least squares means for hip height and wither height through 112 days of age and fecal scores through 56 days of age of calves fed diets containing no additive (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There were no treatment effects (P > 0.05) for hip and wither heights. Treatment effects were observed for fecal score (P < 0.05).

	Treatment					P Value		
	C	YC	P	YCP	SEM[1]	YC	P	YC*P
Hip (cm)	87.22	88.28	87.35	87.53	1.04	0.55	0.76	0.68
Wither (cm)	83.37	84.02	83.20	83.47	1.03	0.66	0.72	0.85
Fecal Score[2]	2.34	2.46	2.39	2.49	0.65	0.02	0.46	0.81

[1]SEM = Standard Error of the Mean

[2]Fecal Score scale: 1=normal; 2=soft; 3=runny; 4=watery

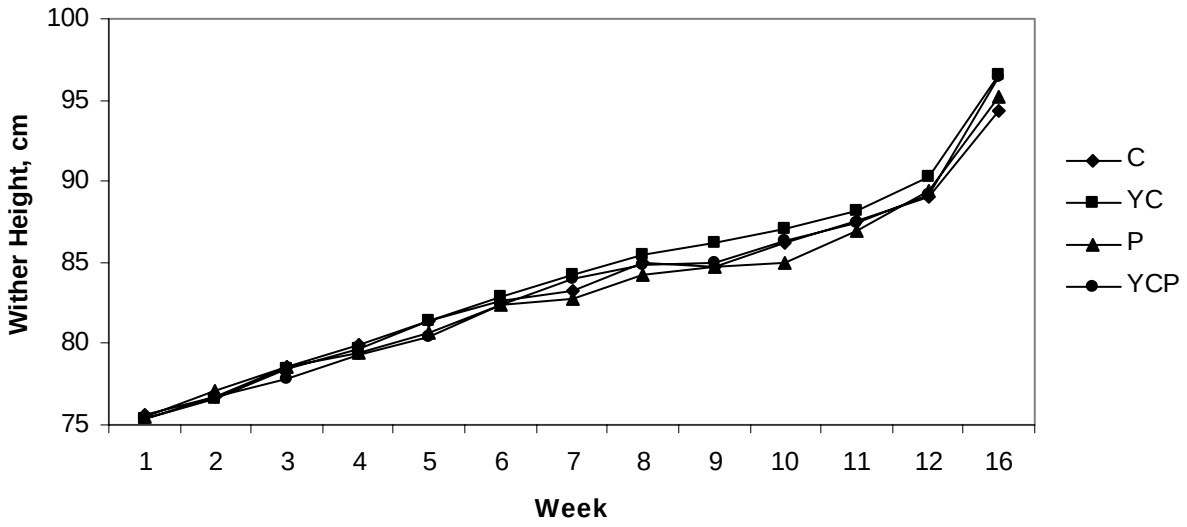

Figure 4. Least squares means of wither height for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a significant time effect ($P < 0.05$). SEM = 0.9530

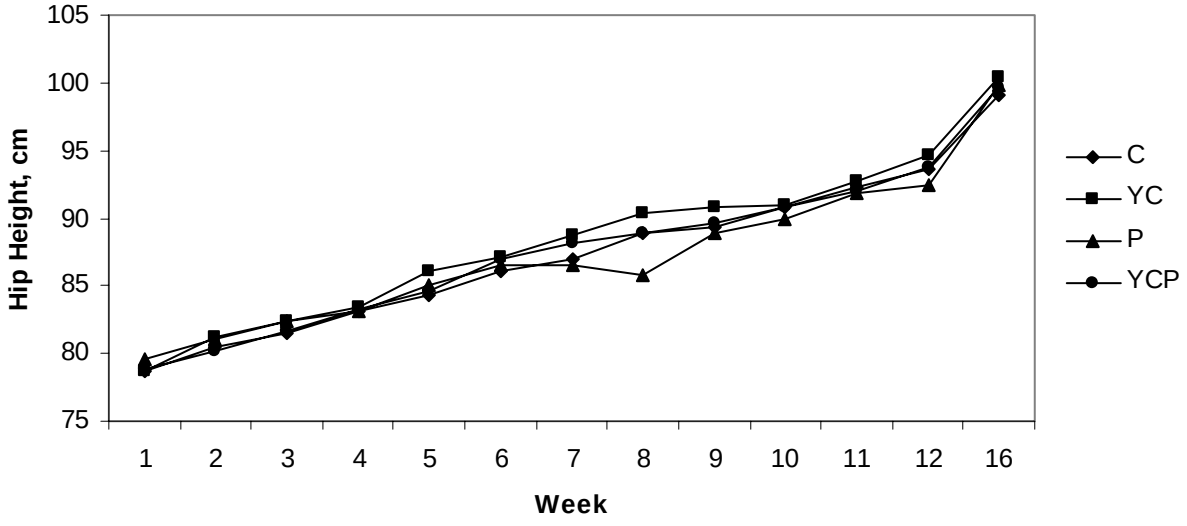

Figure 5. Least squares means of hip height for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a significant time effect ($P < 0.05$). SEM = 0.8857

= 0.0253). However, all fecal scores were well within ranges typically seen in healthy calves, so the biological significance of this effect is minimal. Magalhaes et al. (2008) found that the addition of YC to calf starter significantly improved fecal scores, along with decreasing mortality rates in calves experiencing high incidence of diarrhea. Timmerman et al. (2005) also observed a suppression of diarrhea in calves fed MR supplemented with P. Cruywagen et al. (1995) observed no effect of the addition of P in MR on the occurrence of diarrhea. These additives may improve intestinal health when calves are experiencing problems. But there were no health problems observed in the calves in the current study which may explain the lack of treatment effect on fecal scores.

RUMEN DEVELOPMENT DATA

Least squares means of rumen fermentation parameters for calves fed C, YC, P, and YCP are presented in Table 6. Ruminal pH (Figure 6) was influenced by age of the calf ($P < 0.05$). These results agree with Beharka et al. (1998) who reported a quadratic change in the relationship between pH and age of the calf. Ghorbani et al. (2002) observed no effect on rumen pH in feedlot steers when fed a diet containing P. Quigley et al. (1992) also observed no significant response in rumen pH to YC in calf starter.

Least squares means of NH3 for calves fed C, YC, P, and YCP are presented in Table 6 and Figure 7. No treatment effect was observed, but there was a significant week effect on NH3 concentrations ($P < 0.05$). This result agrees with Vazquez-Anon et al. (1993) who reported a significant age effect of rumen NH3 concentrations with concentrations higher at 4 wk after weaning. However, Anderson et al. (1987) observed higher NH3 concentrations in unweaned calves than in weaned calves. Quigley et al. (1992) reported that the addition of *S. cerevisiae* to calf starter had no effect on NH3 concentrations. Newbold et al. (1996) found that NH3

concentrations increased when mature sheep were fed *S. cerevisiae*, but this result could be attributed to differences in species and age.

Least squares means of plasma BHBA for calves fed C, YC, P, or YCP are presented in Table 6 and Figure 8. No treatment effect was observed, but BHBA levels of calves for all treatments increased over time ($P < 0.0001$). Others have agreed with this increase in BHBA with age (Coverdale et al., 2004; Quigley et al., 1991). Similarly, Lesmeister et al. (2004) did not observe treatment effects in BHBA when calves were fed YC in calf starter. However, Quigley et al. (1992) observed an increase in BHBA when calves were fed YC in calf starter coinciding with an increase in the concentration of butyrate reported.

Least squares means of acetate, butyrate, propionate, and total VFA are presented in Table 6 and in Figures 9, 10, 11, and 12, respectively. There was a significant effect of time for all VFA concentrations ($P < 0.0001$). Other dairy calf studies have also observed this increase in VFA concentrations with age (Beharka et al., 1998; Coverdale et al., 2004). A significant sex*treatment*week interaction occurred for acetate concentration ($P < 0.05$). Calves consuming feed containing P had an increase in acetate, with females showing a greater increase over males. Similarly, Ghorbani et al. (2002) observed no influence of YC on propionate and total VFA, but found that incorporation of P in feed increased the concentration of acetate in feedlot steers. Other studies revealed that YC caused an increase in acetate, butyrate, and total VFA in beef steers (Martin and Nisbet, 1990) and in dairy calves (Quigley et al., 1992).

Table 6. Least squares means of ruminal pH, NH3, and VFA and plasma BHBA for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP) through 112 days of age. There were no significant effects of treatment (P > 0.05).

	Treatment					P Value		
	C	YC	P	YCP	SEM[1]	YC	P	YC*P
pH	6.48	6.40	6.27	6.39	0.07	0.82	0.15	0.20
NH3, mg/dL	6.05	5.76	5.66	6.58	0.75	0.62	0.74	0.35
Acetate, mmol/L	29.11	29.85	31.94	31.79	3.25	0.88	0.24	0.82
Butyrate, mmol/L	4.28	4.65	4.45	4.59	0.56	0.55	0.89	0.79
Propionate, mmol/L	20.48	22.21	23.42	23.41	2.36	0.60	0.21	0.59
Total VFA, mmol/L	58.15	58.09	62.47	62.80	5.28	0.97	0.39	0.97
BHBA, mmol/L	0.25	0.27	0.26	0.26	0.01	0.65	0.84	0.56

[1]SEM = Standard Error of the Mean

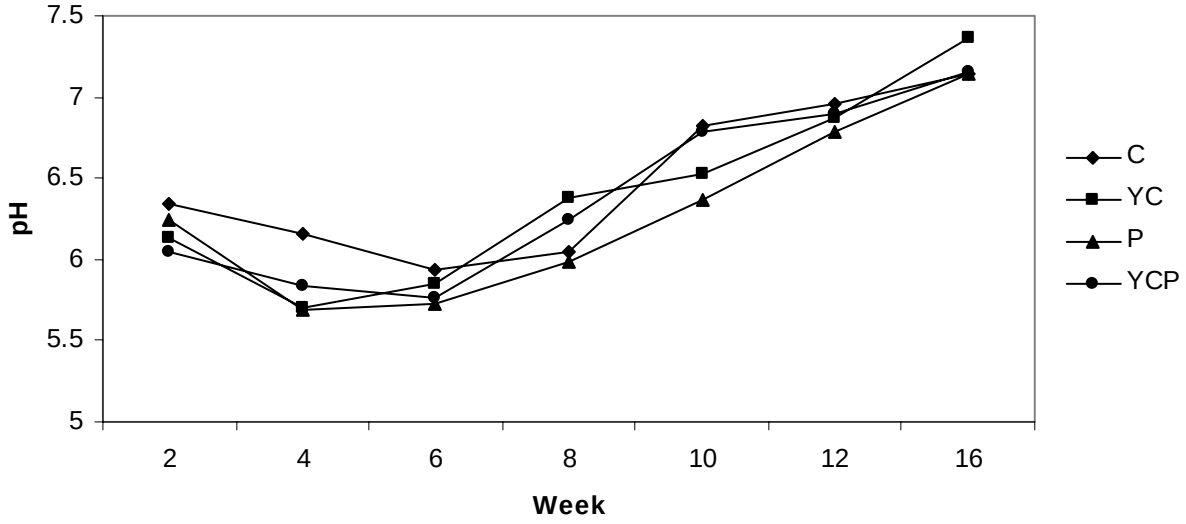

Figure 6. Least squares means of rumen pH for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a significant effect of time ($P < 0.05$). SEM = 0.07764

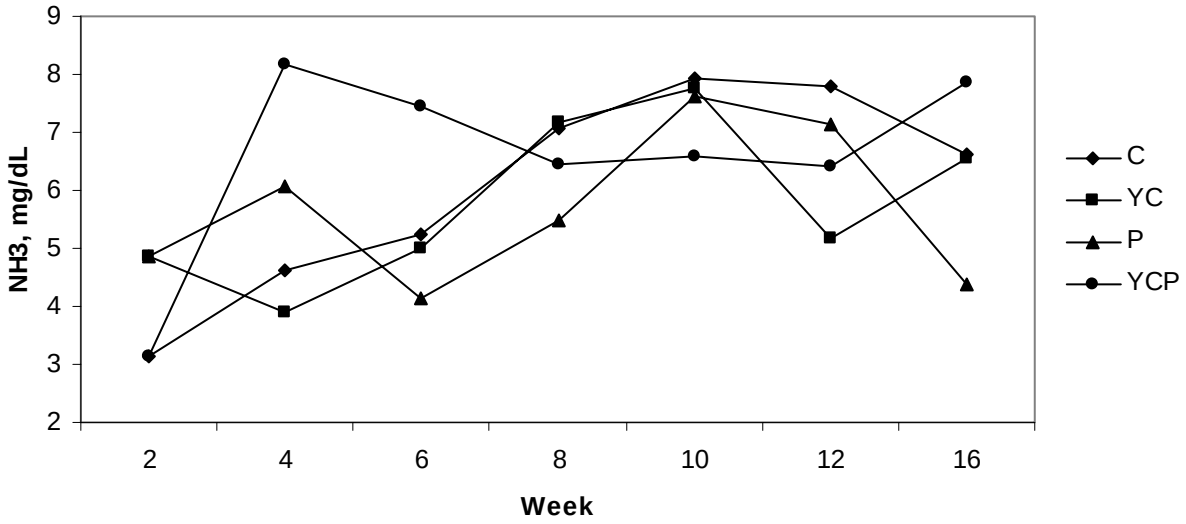

Figure 7. Least squares means of NH3 for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a significant time effect ($P < 0.05$). SEM = 0.6465

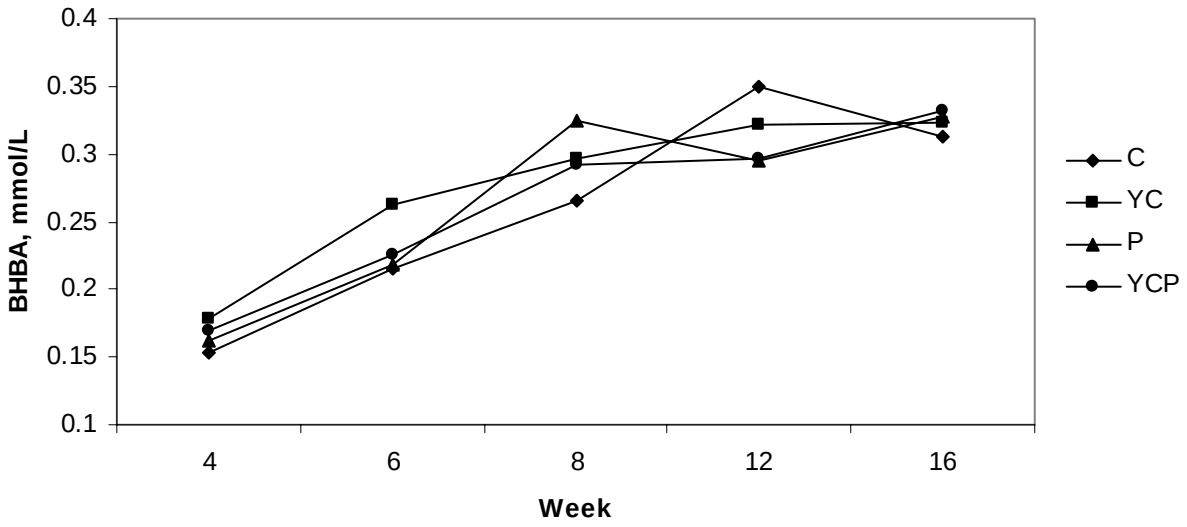

Figure 8. Least squares means of BHBA for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a significant time effect ($P < 0.05$). SEM = 0.01633

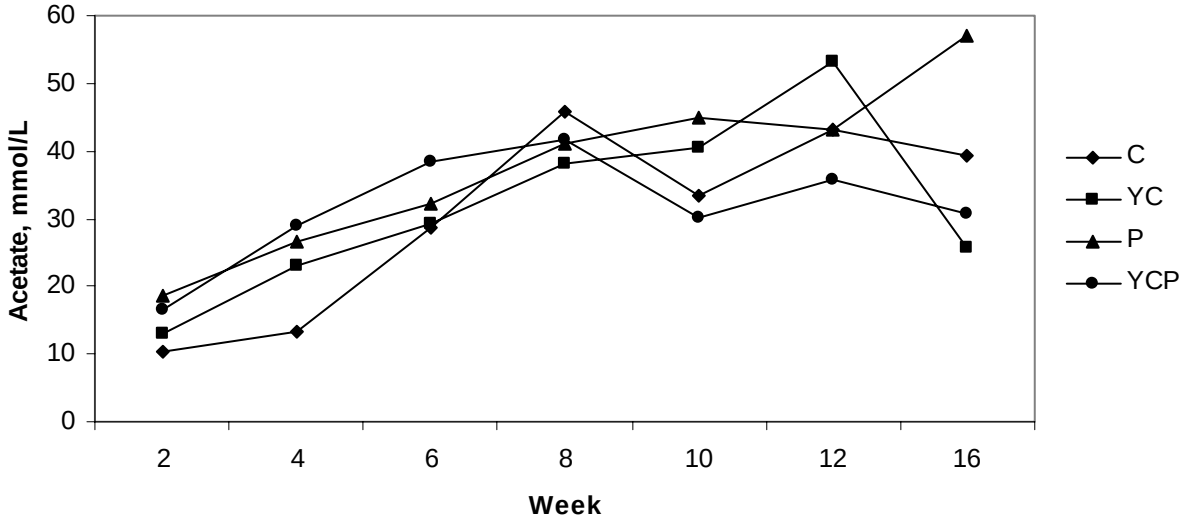

Figure 9. Least squares means of acetate for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a significant time effect ($P < 0.05$). SEM = 2.8337

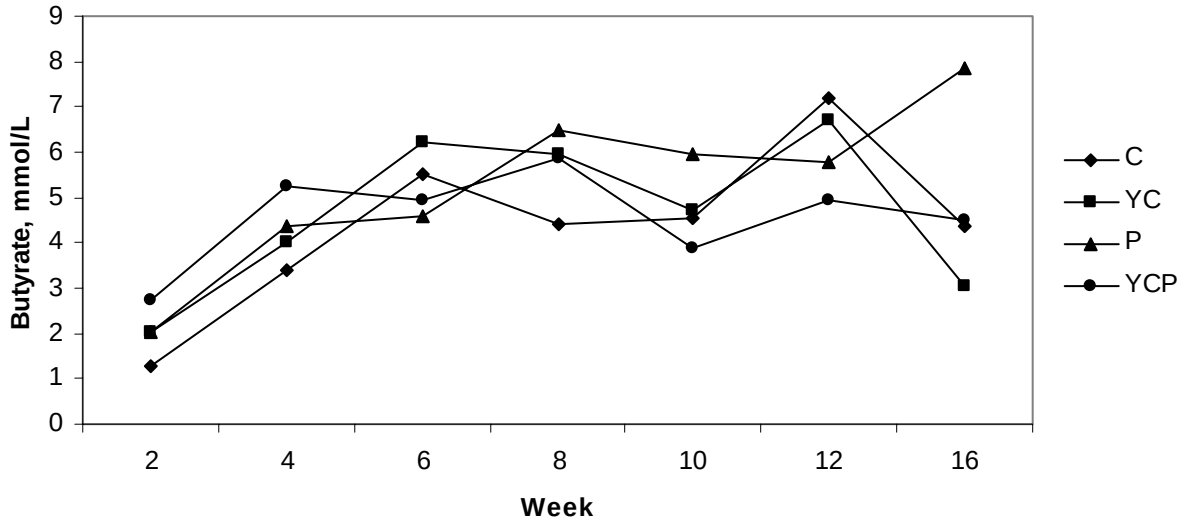

Figure 10. Least squares means of butyrate for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a significant time effect ($P < 0.05$). SEM = 0.4787

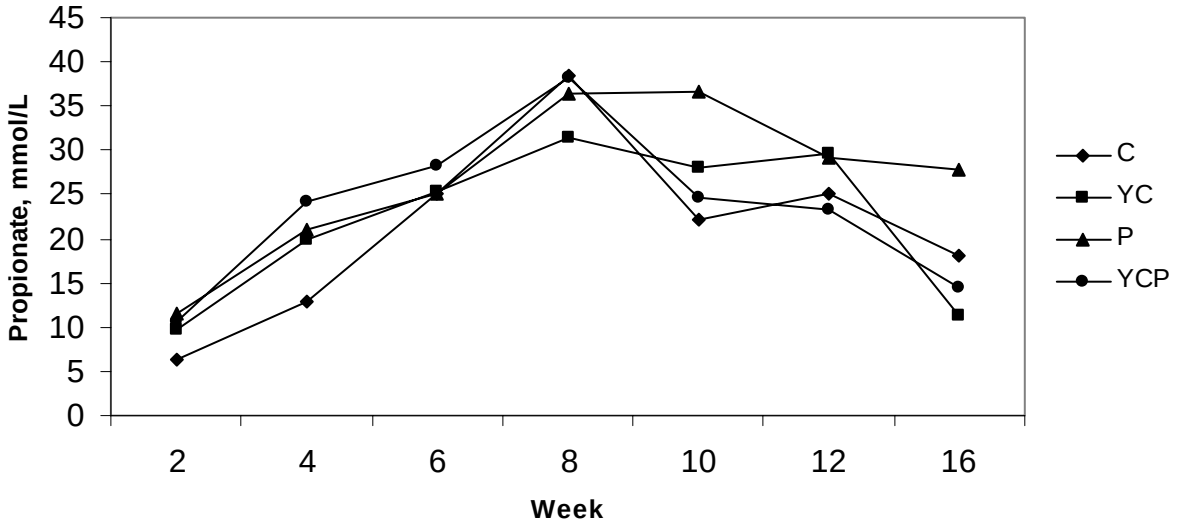

Figure 11. Least squares means of propionate for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a significant time effect ($P < 0.05$). SEM = 2.0744

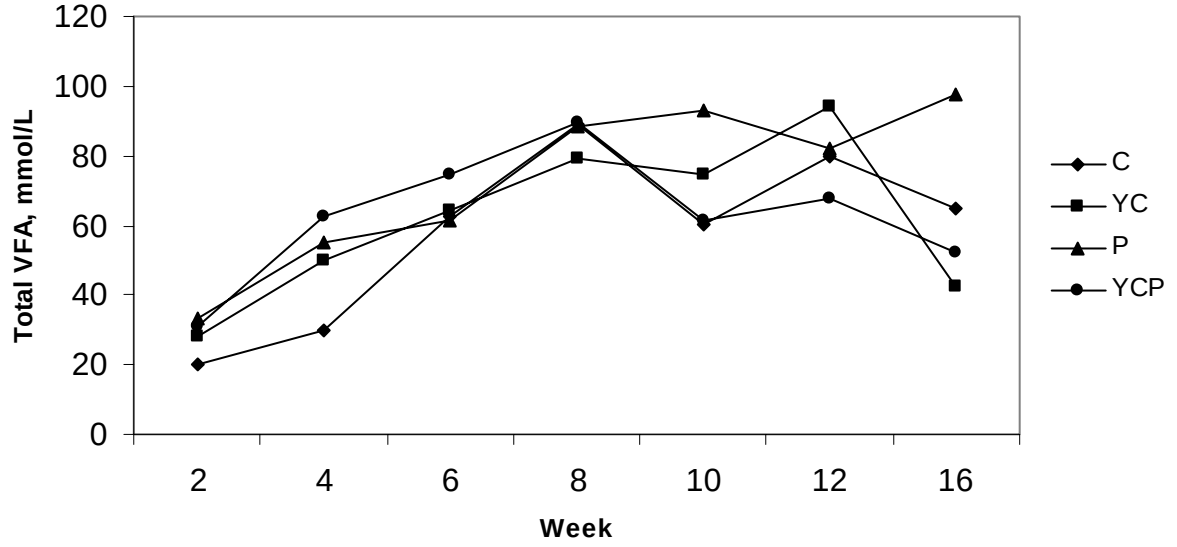

Figure 12. Least squares means of total VFA for calves fed diets containing no additives (C), yeast culture (YC), probiotics (P), or yeast culture and probiotics (YCP). There was a significant time effect ($P < 0.05$). SEM = 5.2889

CHAPTER 5

SUMMARY AND CONCLUSIONS

SUMMARY

A study was conducted to determine the effects of dietary inclusion of probiotics and yeast culture in the diet on rumen development and growth of neonatal Holstein dairy calves. Forty-nine calves (heifers n=20, bulls n=29) were randomly assigned one of four dietary treatments which included calf starter containing no additive (control); calf starter containing the yeast culture *Saccharomyces cerevisiae* at 2% of the dry matter; calf starter containing the probiotics *Bacillus lichenformis* and *Bacillus subtilis* at a minimum total CFU count of 3,20E+09 per gram of product included at a level of 400 g/ton of feed probiotic; and a calf starter containing both yeast culture and probiotics at the above concentrations. Calves were separated from their dams at birth, weighed, and individually housed in 2.5-m² calf hutches with a 2.8-m² wire enclosure on rock bedding until d 56, after which calves were moved to pasture. Calves were offered their treatment diets from day 2 to day 84 d of age.

Body weights were measured at birth and weekly thereafter until d 112 of age. Additionally, wither and hip heights were measured weekly. Feed intake, water intake, and fecal scores were recorded twice daily until d 56 of age. Beginning d 57, calves were group fed their respective treatment at 2,270 g per day per calf and allowed access to ryegrass pasture and free choice grass hay. Rumen fluid was collected biweekly on d 14, 28, 42, 56, 70, 84, and 112 for analysis of pH, short chain VFA, and NH3 to evaluate possible differences in rumen development. Blood was collected on d 28, 42, 56, 84, and 112 for analysis of BHBA concentrations.

Overall mean of average daily intake of starter was not affected ($P > 0.1$) by the incorporation of YC or P in the feedstuffs. Sex by treatment effects were present, but cannot be explained with this study. Overall mean of water intake was significantly affected ($P > 0.05$) by P. Calves consuming calf starter containing P drank less than calves consuming starter without P ($P = 0.0179$). Overall mean fecal scores was affected ($P > 0.05$) by YC. Calves consuming YC had higher fecal scores than those with no YC in their starter ($P = 0.025$). However, all fecal scores were within normal ranges. This is most likely due to the good health of all calves on the experiment.

Overall mean of body weight was unaffected ($P > 0.1$) by treatment. Sex effects ($P < 0.05$) were seen as expected. Again, sex by treatment effects were present, but cannot be explained with this study. Hip and wither height were unaffected ($P > 0.05$) by treatment.

No significant treatment effects ($P > 0.05$) of rumen pH, butyrate, propionate, total VFA, and plasma BHBA were seen. The sex*treatment*week interaction in acetate concentrations cannot be explained with this study and had no biological significance in regard to performance of calves on the study. Week effects ($P < 0.05$) were observed for all parameters and expected.

CONCLUSIONS

Calves consuming calf starter containing YC showed an increase in growth and starter intake at 42 d of age and 56 d of age, but once calves were put into a group feeding situation and given access to forage, this difference was not seen. However, incorporating YC and/or P into feed resulted in no overall effect on growth during the post-weaning period. Though fecal scores for calves consuming YC were slightly higher, it is possible that the addition of YC to calf diets may decrease the incidence of diarrhea during times of stress. Further studies are needed to determine the effects on growth and incidence of diarrhea during times of stress. However,

rumen development remains unaffected overall by the addition of YC and P to grain diets. Proper utilization of feedstuffs remains essential for proper rumen development in the young dairy calf.

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APPENDIX A. β-HYDROXYBUTYRATE COLORIMETRIC ASSAY

(REF: β-Hydroxybutyrate Liquicolor® Procedure No. 2440; STANBIO Laboratory, 1261 North Main Street, Boerne, Texas 78006)

Reagents:

1) Enzyme (R1) (Cat. No. 2441)
2) Catalyst (R2) (Cat. No. 2442)
3) Standard, 1mmol/L (Cat. No. 2443)

Procedure:

1) Incubate the needed amount of Reagent A (Enzyme) at 25°C for 3 minutes.
2) To two cuvettes, add 1075 µL of Reagent A (Cuvettes 1 and 2).
3) To cuvette 1, add 30 µL of sample to be tested and immediately measure the OD at 505nm (To).
4) To the same cuvette 1, add 0.18 mL of Reagent B (Catalyst) and measure the final OD at 505nm (Tf) at 10 minutes.
5) To cuvette 2, add 30 µL of Hydroxybutyrate Standard and immediately measure OD at 505nm (To, std).
6) To the same cuvette 2, add 0.18 mL of Reagent B and measure the final OD at 505nm (Tf, std) at 10 minutes.
1) Subtract To from Tf to obtain OD (10 min) for both serum and standard.

Calculation

$$\beta\text{-Hydroxybutyrate (mM)} = \frac{\text{OD (10 min) Sample}}{\text{OD (10 min) Std}} \times 1\text{mM} \times \text{dilution of serum}$$

APPENDIX B. PHENOL-HYPOCHLORITE ASSAY FOR AMMONIA

Adapted from Broderick and Kang. J. Dairy Sci. (1980) 63:64.

CAUTION: Wear gloves and protective clothing when mixing these reagents of running this assay. Phenol is a cancer-causing agent and will burn the skin. WEAR GLOVES. This procedure allows for the use of repipets or pipetors. After reading, all waste material should be treated as hazardous waste and contained in bottles. All tubes and/or cuvettes must be rinsed before discarding.

Phenol Reagent

Dissolve 0.15g of sodium nitroferricyanide (sodium nitroprusside) in 1.5 L of distilled H_2O (dH_2O). Add 33 mL (90% w/v) phenol (measured in a graduated cylinder) and mix thoroughly. Bring solution to final volume of 3 L by addition of dH_2O and store in brown glass bottle. Phenol needed if 29.7g. Use goggles when measuring phenol and be careful. Phenol can cause burn when it comes into contact with skin.

Hypochlorite Reagent

Dissolve 15g of sodium hydroxide in approximately 2 L of dH_2O. Add 113.6g of disodium phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) to this solution using mild heating and mixing. After the disodium phosphate has mixed, allow the solution to cool. After cooling, add 150 mL of commercial bleach (5.25% sodium hypochlorite, 131.25 mL if using 6% bleach) and mix thoroughly. Bring solution to 3 L by adding dH_2O. Filter solution through #1 filter paper and store in polyethylene bottle protected from light.

Ammonia Standard Solution

A stock solution of 100 m*M* (170mg/dL) ammonia can be prepared by dilution 0.6607g of ammonium sulfate (dry overnight before use) to 100 mL with 0.1N HCl.

Working standards can then be made from the stock solution. Dilute 1 mL of stock solution per m*M* concentration desired in working standard to 100 mL total using dH_2O.

Procedure

1) Sample of ruminal fluid will need to be diluted with dH_2O prior to analysis to bring the concentration of NH_3 into the working range of this assay. Therefore, mix 0.5 mL of clarified ruminal fluid with 4.5 mL of dH_2O and use these samples for the reaction.
2) Add 0.05 mL of sample or standard into test tube (use dH_2O for blanks).
3) Add 2.5 mL phenol reagent to all tubes then mix on vortex.
4) Add 2.0 mL hypochlorite reagent to all tubes then mix on vortex.
5) Place in 95°C water bath for 5 min. Place marbles on top of each tube before inserting in water bath to prevent condensation from falling into the tubes.
6) After cooling, read samples on a spectrophotometer at 630 nm wave-length.
7) Dispose of all waste material in accordance with the hazardous waste regulations of your institution. **This means that the PHENOL cannot be discarded in the municipal sewer without proper authorization.**

APPENDIX C. ANALYSIS OF VOLATILE FATTY ACIDS IN RUMINAL FLUID

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) metaphophoric acid (fluka #79615) acid solution containing 2 g/L of 2-ethyl butyric acid (216.5 µL 2-EB to 100 mL m-phos acid solution; Aldrich #10, 995-9).
2) VFA standard
   a) Add the following volumes of acids to a 100-mL volumetric flask and fill volume with dH_2O. Store in refrigerator when not in use.

MW	Acid	Volume (µL)	Conc (g/L)	Conc (m*M*)
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 stepped 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 providea 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.

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 H_2/ air flame.
6) Helium is used as the carrier gas with a splitless injection at a flow of 60 mL/min.

VITA

Jennifer Marie Laborde was born in August 1983, in Florence, Alabama, to Gerard and Susan Laborde. In February of 1985, she moved to Baker, Louisiana. After graduating from Central High School in 2001, she began her undergraduate studies in animal sciences at Louisiana State University. In May 2006, she received her Bachelor of Science degree in Animal Sciences. After, she began her graduate studies at Louisiana State University in dairy calf nutritional physiology. She will receive the degree of Master of Science in December of 2008.