

**Ruminal colonization and potential of *Megasphaera elsdenii* NCIMB
41125 to stimulate rumen development, and its effects on the
performance of pre-weaned Holstein calves**

By

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Submitted in partial fulfilment of the requirements for the degree

PhD (Animal Science)

**In the Faculty of Natural and Agricultural Sciences
Department of Animal and Wildlife Science**

University of Pretoria

Pretoria

June 2016

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SUMMARY

Ruminal colonization and potential of *Megasphaera elsdenii* NCIMB 41125 to stimulate rumen development, and its effects on the performance of pre-weaned Holstein calves

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Four studies were conducted to determine the survival of *Megasphaera elsdenii* NCIMB 41125 in the gastro intestinal tract (GIT) of Holstein calves after an oral dose, and evaluate its effects on ruminal fermentation, rumen development and performance. The objective of the first study was to investigate the survival of the bacteria in the GIT and evaluate effects on rumen fermentation and blood metabolites. The second study evaluated the effects and dosing time on intake, growth and diarrhoea in a conventional calf growth system. The third study evaluated the pre- (d 1-56) and post-weaning (d 56-70) effects of the bacteria in an accelerated growth system on intake, growth and blood beta-hydroxybutyrate (BHBA). The fourth study evaluated the effects on rumen development and fermentation. In experiment one, a higher cell count of *M. elsdenii* (24 h after dosing) and *M. elsdenii* NCIMB 41125 (24 and 72 h after dosing) were found in the rumen and the colon after dosing on day 7 and 14. An increase in the proportion of butyrate was observed 72 hours later. In experiment 2, measured for 42 days after birth, dry matter and water intakes were increased ($P < 0.05$) after dosing with *M. elsdenii* NCIMB 41125 on days 7 and 14 when compared to control calves. Feed efficiency, however, was not affected by dosing. The average numbers of days that diarrhoea was observed were 18 and 4.5 % less in calves dosed on d 7 and d 14, and 11.4 % higher in those dosed on d 21 compared to control calves. In the third study, dosed calves had higher starter DMI compared to control calves ($P < 0.01$). The opposite was true for milk intake as dosed calves consumed less milk ($P = 0.01$) than control calves. The total DMI, energy intake

and ADG were not affected ($P > 0.05$), but dosed calves were 5.8 kg heavier ($P = 0.01$) at weaning and had greater gain:feed ratio ($P < 0.01$) compared to control calves. The plasma BHBA concentration after dosing was greater for dosed calves ($P = 0.02$) compared to control calves. After weaning, dosed calves consumed more ($P < 0.05$) feed and energy, gained 0.37 kg more weight per day ($P < 0.01$), and weighed 11.6 kg more ($P < 0.001$) at the end of the study compared to control calves. In the last experiment, dosed calves had a higher reticulo-rumen weight ($P = 0.01$), papillae width ($P < 0.001$) and papillae density ($P = 0.02$) compared to control calves. The rumen wall thickness and papillae length were not affected by dosing. Total VFA's, acetate and propionate were also not affected, but butyrate concentration was higher in dosed compared to control calves ($P < 0.04$). These results indicate that *M. elsdenii* NCIMB 411 can colonize and establish in the GIT of pre-weaned calves after a single dose of the bacteria. The increased starter intake, plasma BHBA, and improved rumen fermentation and development in dosed calves suggest greater metabolic activity of the rumen epithelium. Benefit of dosing on feed efficiency was observed on calves in accelerate growth and early weaning.

ACKNOWLEDGEMENTS

My first and ultimate thanks go to my Heavenly Father for all of the wonderful blessings he has sent my way. I know that God has led me to this point in my life and it is only his grace that has brought me to where I am today.

My wife Monique Kalala Mbuyi, my children Noemi, Hervine and Olivia for your love, prayers, support and patience.

My sincerest thanks and gratitude to:

Prof Lourens Johannes Erasmus, for the guidance and support.

Dr Florence Nherera for your advices and support

Sandra Erasmus and the technical team of the dairy section of the Agricultural Research Council for your contributions to my research work.

Bob Tshitungi Muya, my brother for your continuous encouragement and moral support
ARC for the funding.

DECLARATION

I, Claude Mukengela Muya declare that the thesis, which I hereby submit the degree PhD (Animal Science) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature

Date:

List of conference proceedings and published papers:

Muya M. C., L. J. Erasmus, K. A. Miller, C. C. Aperce, F. V. Nherera and P. M. Moshidi. 2016. Performance of Holstein calves having free access to milk and dosed with *Megasphaera elsdenii*. Submitted (accepted) for publication to the Scientia Agricola Journal. May 2016.

Muya M. C., F. V. Nherera, K. A. Miller, C. C. Aperce, P. M. Moshidi and L. J. Erasmus. 2015. Effect of *Megasphaera elsdenii* NCIMB 41125 dosing on rumen development, volatile fatty acid production and blood b-hydroxybutyrate in neonatal dairy calves. Journal of Animal Physiology and Animal Nutrition. 99 (5): 913–918.

M. C. Muya, F.V. Nherera, K. A. Miller, C. C. Aperce, P.M. Moshidi, and L. J. Erasmus. Effect of *Megasphaera elsdenii* NCIMB 41125 dosing on rumen development, volatile fatty acid production and blood β -hydroxybutyrate in neonatal dairy calves. Symposium on Gut Health in Production of Food Animals. November 10-12, 2014, Saint Louis, Missouri. United States.

M. C. Muya, K. A. Miller, C. C. Aperce, and L. J. Erasmus. Performance and rumen development of Holstein calves dosed with *Megasphaera elsdenii* NCIMB 41125. Symposium on Gut Health in the Production of Food Animals. November 11-13. November 2013. Marriott Kansas City Country Club Plaza, Kansas City, Missouri, United States.

F.M. Hagg, M.C. Muya and P.H. Henning. 2010. The effect of *Megasphaera elsdenii* NCIMB 41125 (ME) on performance of pre-weaned dairy calves. J. Anim Sci; 88:E-Suppl. (2):420.

List of abbreviations

ADF: Acid detergent fiber

ADG: Average daily gain

AOAC: Association of official analytical chemists

BHBA: Beta-hydroxybutyrate

BW: Body weight

CP: Crude protein

DFM: Direct fed microbial

DM: Dry matter

DMI: Dry matter intake

GE: Gross energy

GIT: Gastro-intestinal Tract

ME: Metabolize energy

NDF: Neutral detergent fiber

NFC: Non fiber carbohydrate

NRC: National Research Council

NSC: Non-structural carbohydrate

OM: Organic matter

PCR: Polymerase chain reaction

SCFA: Short chain of fatty acid

SEM: Standard error of the mean

TCA: Tri-carboxylic acid

VFA: Volatile fatty acid

CHAPTER 1.

GENERAL INTRODUCTION

The rumen of the neonatal calf undergoes both physical and metabolic development, following the initiation of solid feed intake and the subsequent establishment of ruminal fermentation and microbial population. Physical development of the rumen consists of increased mass and growth of the papillae. It is known that physical stimulation by feed in the rumen accounts for measurable increases in both weight and musculature development, and that the presence of physical bulk does not, however, stimulate papillary development (Hamada et al., 1976). Thus, for normal development of the ruminal epithelium to progress, viable ruminal fermentation must take place, suggesting that there is a requirement for the presence of short chain fatty acids to initiate normal papillary development (Sander et al., 1959).

New-born dairy calves are separated from their dams and fed milk replacers or whole milk and offered calf starter. The amount of milk consumed by young dairy calves influences gut development (Anderson et al., 1987) and determines intake of starter feed as well as their health and growth (Appleby et al., 2001). In conventional calf-rearing systems, the amount of milk or milk replacer is limited to 8-10 % of body weight (BW) during the first few weeks of life (Drackley, 2005) in order to encourage solid dry matter intake (DMI) and allow early weaning. Studies to improve milk feeding systems for dairy calves through *ad libitum* milk feeding have shown higher milk consumption and BW gain, with reduced starter intake, compared to restricted milk feeding (Appleby et al., 2001; Hammon et al., 2002; Jasper and Weary, 2002). Delayed solid feed intake because of *ad libitum* milk consumption during the pre-weaning period (Appleby et al., 2001; Hammon et al., 2002) results in delayed ruminal development which is associated with poor post-weaning performance (Baldwin et al., 2004).

The concept of ad lib milk feeding or accelerated growth feeding systems as it is known in the United States, has been successfully adapted by many farmers (Jasper and Weary, 2002; Drackley, 2008; Khan et al., 2011, Stamey et al., 2012). Under these systems, calves can reach average daily gains (ADG) of up to 1.0 kg/d during the pre-weaning period. The concentration of fat and protein in the milk replacer fed in these systems are around 15-22 % and 28 % respectively and milk can be fed up to 20 % of BW (Cowles et al., 2006). Under these systems, starter feed intake, however is not sufficient (Jasper and Weary, 2002; Uys et al., 2011).

It is well known that rumen development is an important factor in determining early solid feed intake and performance in calves (Gorka et al., 2009). Dairy producers and nutritionists should therefore focus more on early rumen development as a mean to improve solid feed intake. The role of feed additives to enhance rumen papillae development, for example, is an aspect that deserves research attention.

Apart from the fact that improved rumen development increases solid feed intake, it is vital for the calf to make a smooth transition from a pre-ruminant to a functioning ruminant, and that could allow producers to wean younger calves that have more mature digestive systems (Coverdale et al., 2004). In addition, there is a positive correlation between pre-weaning calf growth and first lactation performance (Soberon et al., 2012; Soberon and Van Amburgh, 2013). Soberon and Van Amburgh (2013) reported that for every pre-weaning kg of weight gain there was 1.5 kg more milk during their first lactation. A popular trend in some regions of the world towards early weaning of newborn dairy calves necessitated investigating new ways of accelerating the gastrointestinal tract development (Guilloteau et al., 2009).

Total nutrient intake decreases immediately after weaning and this may potentially expose animals to stress and reduced resistance to infection. Weaning based on solid feed consumption or progressively decreasing milk allowance has been suggested as strategies to minimize reduction in nutrient intake at weaning. Fostering solid feed intake, in addition to the starter feed, by offering chopped straw or poor quality hay to calves has also been proposed as an alternative (Castells et al., 2012).

Butyrate and propionate are the primary volatile fatty acids responsible for rumen epithelial and papillae development in the pre-weaned ruminant (Tamate et al., 1962; Coverdale et al., 2004). Increasing the proportions of butyric and propionic acids, increases blood flow to the ruminal epithelium, which stimulates vascular budding and epithelial cell proliferation. Papillae either grow in size and number or shorten in size, depending on the diet and the fermentation acids produced.

Microbial feed additives that enhance health and performance of young calves are continuously being evaluated (Heinrichs et al., 2003; Krehbiel et al., 2003; Hong et al., 2005; Seo et al., 2010). During early life, when milk is the main ingredient of the diet, the rumen tissue structure is undeveloped and food tends to bypass the rumen. The first function of microbial feed additives during this time, for example *Lactobacillus acidophilus*, is the prevention of diarrhoea by modifying the flora of the small intestine.

Megasphaera elsdenii, a gram negative coccus, is by far the most significant of lactic acid-utilising bacteria found primarily in calves and cattle receiving rations high in starch (Stewart & Bryant 1988). It can also be found in intestinal contents of humans and pigs (Giesecke et al., 1970; Marounek et al., 1989). *Megasphaera elsdenii* is thought to play a major role in production of branched-chain volatile fatty acids in the rumen. The mechanism involves deamination and decarboxylation of amino acids (Allison 1978; Wallace 1986). The primary function of *M. elsdenii*, however, is to convert lactic acid to different VFA's, i.e. propionate and butyrate and to convert glucose to butyrate (Marounek et al., 1989).

Megasphaera elsdenii NCIMB 41125, a strain isolated from feedlot beef cattle and lactating dairy cows, was found to be efficient against lactic acid accumulation in the rumen of cattle (Kettunen et al., 2008; McDaniel et al., 2008; Henning et al., 2010b). *Megasphaera elsdenii* NCIMB 41125 improved ADG in steers (Henning et al., 2009) and increased feed intake in lambs (Henning et al., 2010a).

No literature could be found where *M. elsdenii* was supplemented to young calves with the purpose of early rumen development. The objectives of our study therefore were to determine whether *M. elsdenii* could colonize the rumen, stimulate rumen development, increase starter feed intake and thereby increase calf growth performance under both conventional and accelerated growth systems. The objectives are discussed in more detail in Chapter 3, following the literature review in Chapter 2.

CHAPTER 2.

LITERATURE REVIEW: Calf feeding systems, feed supplements and rumen development.

2.1. Feeding systems in calf rearing

Depending on the targeted growth rate and weaning age of calves, a predetermined liquid feeding program can be adopted. Young dairy calves can be fed a limited (generally 10 % of calf BW) amount of milk or milk replacer (conventional system) with calf starter *ad libitum* to stimulate solid feed consumption (Drackley 2005). However, calves can also be fed a high volumes of milk or milk replacer, up to 20 % of BW to improve growth (accelerated growth system). Calves can also be raised in groups, which stimulate calf contact and improve welfare (European Council, 1997). When reared by the dam, the calf initiates suckling from the cow within a few hours after birth, and will suckle its dam several times (Reinhardt and Reinhardt, 1981), and can consume about 9 kg/d within the first week (de Passillé et al., 2008), with the frequency and suckling time declining as the calf ages (Nolte et al., 1990).

2.1.1. Conventional calf feeding system

For years research has focused on developing feeding management strategies that promote early weaning and transitioning calves from milk to concentrate feed (Williams and Frost, 1992; Baldwin et al., 2004) with the objective of improving calf starter intakes by reducing the amount of milk fed to calves. In a conventional system, calves are separated from their dams and fed limited amounts of milk, using buckets or nipples in order to encourage solid feed intake and allow early weaning (Drackley 2005). In a typical conventional feeding system Holstein calves will be fed at 10% BW, therefore 2 litres of milk twice daily with calf starter *ad lib* from the third day (Jasper et Weary, 2002). Colostrum will be fed the first 1 to 3 days. This restricted milk feeding is reported to be associated with depressed performance due to low nutrient availability (Appleby et al., 2001), poor welfare (Khan et al, 2007) and health (Huber et al., 1984), and low productivity (Pollack et al., 1993). In this system, growth rates are low compared with calves reared by the cow (Flower and Weary, 2001), and low nutrient intake may contribute to high rates of calf mortality and morbidity (NAHMS, 2007). Borderas et al. (2009) reported an ADG of 0.48 kg/d during the first 21 d and 0.80 kg/d between day 22 and

weaning (50 d) for calves fed 4L/d of milk. The calculated average for the whole period was 0.66 kg/d. When raised in group, Uys et al. (2011) reported that calves raised in small (n=15) and large (n=30) groups, and fed restricted milk for weaning at 42 d gained 0.59 and 0.57 kg/d, respectively, compared to similar groups fed high milk volumes (0.75 and 0.71 kg/d). Calves receiving milk or milk replacer at 10% BW can consume up to twice the amount of starter consumed by calves on enhanced programmes (Nielsen et al., 2008).

Although this restricted feeding system of raising calves allows producers to wean healthy calves earlier (less than 6 week of age) and at low cost, Diaz et al. (2001) demonstrated that following a conventional feeding program calves needed an additional 28 days to reach 85 kg of BW compared to calves following an accelerated-growth feeding program (Diaz et al., 2001).

2.1.2. Accelerated growth calf feeding system

Calves fed milk ad libitum (von Keyserlingk et al., 2006) or suckling their dam (Bar-Peled et al., 1997) generally achieve higher growth rates. A study by Khan et. (2011) demonstrated that calves fed ad lib milk can consume up to 19 % of BW and gain approximately 1 kg/d, with a gain:feed ratio of 0.81. Jasper and Weary (2002) reported that ad lib milk fed calves consumed about 90 % more milk, with a 63 % higher ADG than calves in conventional system (10% of BW). Increasing metabolisable energy intake is required to support increasing ADG. When feeding milk replacer, a more nutrient dense powder is required to provide enough energy. Holstein calves fed 10 %, 14 %, or 18 % of BW of a 26 % CP milk replacer, gained 0.36, 0.70 and 1.02 kg/d, respectively (Bartlett, 2001). Protein requirement is a function of the energy allowable gain (Davis and Drackley, 2000; Van Amburgh and Drackley, 2005). As the energy intake increases the protein required to meet the energy allowable gain increases (Table 2.1).

Table 2.1. Protein requirements of pre-weaned dairy calves as influenced by the rate of body weight gain with constant initial body weight (45.3 kg) (adapted from Davis and Drackley, 1998; From Drackley, 2000)

Rate of gain (kg/d)	ME, (Mcal/d)	Required DMI (lib/d)	CP required, (% of DM)
0	1.75	0.38	8.3
0.45	3.01	0.66	22.9
0.91	4.64	1.02	26.6
1.36	6.46	1.42	27.6

ME: Metabolisable energy; DMI: Dry matter intake; CP: Crude protein

However, feeding high amounts of milk increases growth slump at weaning (Khan et al., 1980). For years, the fear of feeding milk replacers with high dry matter concentrations was the potential risk of causing diarrhea. It was concluded after several observations (Hammon et al., 2002) that higher incidences of diarrhoea is usually related to sanitary, management, and housing conditions of the calves rather than their daily amounts of milk intake. Greater amounts of milk fed to calves did not cause any noticeable increase in diarrhoea (Appleby et al., 2001; Jasper and Weary, 2002; Khan et al., 2007). Feeding milk *ad lib* may also result in increased fat deposition (Diaz, et al., 2001; Bartlett et al., 2006), impairing development of the mammary gland (Silva et al., 2002). This problem can be avoided by increasing protein intake and following NRC recommendations. There are numerous examples where accelerate growth systems were implemented successfully with significantly increased feed efficiencies and ADG's (Jasper and Werry, 2002; Shamay et al., 2005).

2.1.3. Calf immune system

The neonatal calf immune system is immature and more susceptible to infectious diseases the first days after birth (Hauser et al., 1986) and the calf rely on the passive immunity conferred by the colostrum. Ensuring adequate intake of colostrum to support the calf's passive immunity is the key to successfully calf rearing. Tyler et al. (1996) and Berge et al. (2005) classified serum IgG level of ≥ 1 mg/dl as adequate passive transfer. Calves with failure of passive transfer have higher mortalities and morbidity and need more antibiotic treatments compared to animals with adequate passive transfer of immunity (Berge et al., 2005). Feeding antibiotics is widely used in calf rearing as a strategy to improve serum immunoglobulin levels (Quigley et al., 1997; Constable, 2003). The calf immune system differs from that of adult animals in terms of the peripheral blood mononuclear cells population, and occurrence of neutrophils (Hauser et al., 1986). Furthermore, the classical and alternate pathways of

complement activation (Mueller et al., 1983) activities are reduced in the calf immune system. Higher plasma insulin-like growth factor I (IGF-I) concentrations were found in calves fed high amounts of milk replacer compared with calves fed conventional amounts (Smith et al., 2002). Accelerated-growth feeding programs may benefit the immune system of the neonatal calf because it increases plasma insulin-like growth factor I (IGF-I) concentrations. This growth factor plays an important role as a cell proliferation cofactor and differentiation factor on B lymphocytes development and it enhances the proliferative response of lymphocytes to mitogens (Nonnecke et al., 2003).

2.2. Direct fed microbials in ruminant feeding

2.2.1. Type of direct fed microbial

Direct fed microbials (DFM) have been used in the cattle industry for many years with the objective of improving performance (LeJeune and Wetzel 2007) and reduce the prevalence of pathogens in livestock. Different mechanism have been suggested whereby DFM improve performance. The first concept of feeding bacterial DFM to livestock was based on beneficial post-ruminal effects (Nocek et al., 2002); however, a number of ruminal effects have been indicated, particularly, prevention of ruminal acidosis. Lactate-producing bacteria (*Lactobacillus* and *Enterococcus*) have been reported to help prevent ruminal acidosis in dairy cows (Nocek et al., 2002), by causing the microorganisms present in the rumen to adapt to the presence of lactic acid in the rumen (Yoon and Stern, 1995). Inoculation with *Megasphaera elsdenii* has been shown to prevent lactate accumulation when a highly fermentable substrate was used (Kung and Hession, 1995; Hagg et al., 2010). Enhancing the growth of strict anaerobes that become predominant in the adult rumen and to establish as rapidly as possible a healthy, fibre-digesting fermentation in the rumen was also suggested as one of the objective for feeding DFM's, especially yeast products (Wallace and Newbold, 1992).

Direct binding to the intestinal epithelium and in some instances alteration of gene expression in targeted pathogens (Medellin-Pena and Griffiths 2009) are mechanisms whereby DFM may exclude pathogens. The alteration of gene expression was recently demonstrated when a cell-free medium from *L. acidophilus* (strain La-5) inhibited the colonization of specific-pathogen-free mice by *E. coli* O157: H7. In calf rearing, DFM are supplemented as feed additives, beneficially affecting the host by improving its intestinal microbial balance (Fuller, 1989).

These additives are frequently added to milk or milk replacer to improve gastrointestinal health, growth rate, daily feed intake, and feed efficiency (Fuller, 1989). The usage of DFM as supplements for calves was reported to have increased from 13.1 % to 20.0 % from 1996 to 2007 (USDA, 2008).

Viable cultures of fungi and bacteria are microorganisms used as DFM's for ruminants to potentially replace or reduce the use of antibiotics in neonatal and stressed calves, to enhance performance in dairy cows, and to improve efficiency of feed conversion and ADG in beef cattle. Basic mechanisms of the effects of DFM's are still to be understood (Krehbiel et al., 2011). Functions of probiotics include protecting young animals against enteropathic disorders and improving feed conversion efficiency and weight gain in growing animals (Windschitl et al., 1991). Commonly used probiotics include, amongst other, *Lactobacilli*, *Streptococci*, *Enterococci*, *Bifidobacteria*, and *Propionibacteria* (Walker, 2007). Most of these have been shown to be the most active in the lower gut of ruminants.

2.2.2. Mode of action of direct fed microbials

2.2.2.1. Bacteria, protozoa and fungi

Amongst the large micro-organism community in the rumen, most approaches in the use of DFM have focused on the use of rumen bacteria to alter the profile of fermentation products (Jones et al. 2009). A few studies have explored the extent to which inoculation with ruminal fungi may improve fiber digestion (Sehgal et al. 2008). Some studies have attempted to reduce the amount of lactic acid produced by introducing rumen bacteria (*Prevotella bryantii* 25A) that utilize starch, but produce fermentation end products other than lactic acid (Chiquette et al. 2008). Others have aimed at enhancing ruminal lactic acid metabolism through inoculation with lactic acid utilizing bacteria (Wiryawan and Brooker 1995; Klieve et al. 2003; Raeth-Knight et al. 2007). Although DFM are derived from the rumen, cultured rumen bacteria frequently fail to persist in the rumen since they undergo morphological and metabolic changes (Stewart et al. 1997). The commercialisation of rumen-based DFM was challenged in the past due to the specificity of media required for the growth of rumen microbes, exclusion of oxygen during packaging and storage and oxygen sensitivity precluding the administration of anaerobic DFM within feed (Krause et al., 2001).

2.2.2.2. Yeast products

Yeast products, mostly different strain of *Saccharomyces cerevicae* can be fed as either yeast culture, live yeast or rumen specific live yeast. *Saccharomyces cerevisiae* has been used extensively in dairy cattle for improving efficiency through increased dry matter intake, stabilizing of rumen pH, increased rumen volatile fatty acids production (VFA) and organic matter digestibility, as well as decreasing rumen lactate concentration, especially when fed higher levels of concentrate (Desnoyers et al., 2009). *In vitro* supplementation with live yeast stimulated growth and activities of some ruminal fiber-degrading microorganisms (Chaucheyras- Durand et al. 2008). It was proposed that the increased fiber degradation is mediated through the ability of yeast to scavenge oxygen within the rumen (Newbold et al. 1996). This theory is supported by the lower redox potential of ruminal fluid in cows in the presence of live yeasts (Marden et al. 2008), indicating that yeast can strengthen the reducing power of ruminal fluid. Yeast has also resulted in alteration of rumen VFA proportions. While Newbold et al. (1995) reported increased propionate at the expense of acetate with yeast supplementation, some authors reported increased acetate (Mutsvangwa et al., 1992; Mwenya et al., 2005), while others found no effects on ruminal VFA (Dawson et al., 1990; Piva et al., 1993; Moallem et al., 2009). Increasing concentrations of live yeast in *in vivo* study showed no effect on molar proportion of acetate in one study (Lila et al., 2004) and increased effect in another (Lila et al., 2006). Because of the inconsistency of the effects of yeast culture on ruminal VFA, Wallace and Newbold (1992) concluded, that it is unlikely that the production benefits seen when yeast is added to the diet arise from changes in the stoichiometry of VFA formation. Supplementation with live yeast tended ($P < 0.10$) to decrease ruminal Acetate:Propionate ratio (Martin et al. 1989). Jouany et al (1999) reported that the shifts in bacterial populations observed with *S. cerevisiae* reflect the ability of yeast to utilize the trace amounts of oxygen present in the rumen, thereby creating an environment that is more conducive for the activity of anaerobic cellulolytic bacteria. Robinson and Erasmus (2009) suggested that yeast cultures contains micronutrients responsible for the growth of some rumen microbes, thereby altering the pattern of rumen fermentation.

In calves, yeast culture has been shown to improve growth performance and health of calves when supplemented in the milk replacer (Linn and Raeth-Knight, 2006). Fed to calves from day 2 to weaning at day 56, yeast culture tended to increase DMI. Body weight was increased two weeks before weaning, but rumen development was not affected (Laborde, 2008).

2.2.2.3. Lactic acid-producing bacteria

Direct fed microbial supplements include in many instances one or more lactic acid-producing bacteria and are most often supplemented in beef and dairy as part of the premix. Lactic acid-producing bacteria are desirable as DFM as they lend themselves to industrial culture, are environmentally robust and have a number of mechanisms whereby they may alter or influence microbial communities.

Bacterial DFM, which include species of *Lactobacillus*, *Enterococcus*, *Streptococcus*, and *Bifidobacterium*, have been studied in experiments with young calves (Newman and Jacques, 1995). *Lactobacillus acidophilus* has potential to promote ruminal development of Holstein calves as indicated by more rumination at 30 days by calves supplemented with *L. acidophilus* compared to untreated calves (Nakanishi et al., 1993). However, results have been inconsistent. While some researchers (Bechman et al., 1977; Beeman, 1985) reported improved growth rates, others (Ellinger et al., 1978; Abu-Tarboush, 1996) reported no change in weight gain as a result of feeding *L. acidophilus*. In suckling calves, lactic acid-producing bacteria are often administered as a bolus, but sometimes associated with a carrier, whereas in adult cows they are more commonly administered through the diet.

Inoculation of lactic acid-producing bacteria onto forages before ensiling is used as a means of enhancing the preservation, feed value and aerobic stability of silage (Kang et al. 2009; Schmidt et al. 2009). Lactic acid-producing bacteria produce not only lactic acid, their main antimicrobial compound, which disrupt the intracellular pH of bacterial competitors, but also produce bacteriocins (Servin 2004). These bacteria also produce, in the presence of oxygen, hydrogen peroxide, which is believed to limit Salmonella activity (Pridmore et al. 2008). Some lactic acid-producing bacteria produce other compounds such as benzoic acid, diacetyl, mevalonolactone, methylhydantoin and reuterin (Brashears et al. 2005).

Lactic acid-producing bacteria are often administered in combination with other bacteria or yeast to exploit potential complementarity or additive effects. In the past, combination of bacteria such as *Bacillus* spp. and *Bifidobacterium* spp. have been used as DFM (Flint and Garner 2009). *Bacillus* spp., however are often present in low numbers in the rumen, (Oyeleke and Okusanmi 2008). In monogastrics, *Bifidobacterium* spp. colonize the intestinal tract shortly after birth (Lie´vin-Le Moal and Servin 2006) and play a key role against enterovirulent

microorganisms involved in the occurrence of diarrhea (Servin 2004). In the rumen, *Bifidobacterium* spp. most likely plays a role in starch digestion. Their role in the metabolism of sugars in the lower intestinal tract is less pronounced than in monogastrics (Stewart et al. 1997). In calves, *Bifidobacterium* spp. was included as a DFM along with other microbial species (Krehbiel et al. 2001).

2.2.2.4. Lactic acid utilising bacteria

Several studies have shown enhanced ruminal metabolism of lactic acid through inoculation with bacteria such as *M. elsdenii*, *S. ruminantium* and *P. freudenreichii* (Wiryanawan and Brooker, 1995; Klieve et al., 2003; Raeth-Knight et al., 2007). An alternative approach of reducing ruminal lactic acid produced is to inoculate with specific strain of bacteria such as *Prevotella bryantii* that utilize starch and then produce other end products of fermentation than lactic acid (Chiquette et al. 2008).

Direct fed microbials might reduce the risk for subacute acidosis by reducing the time ruminal pH remains below 5.6. Lower blood CO₂ and layered double hydroxide (LDH) also suggest a lower risk for metabolic acidosis. However, these responses seem to depend on the species of DFM fed. Propionibacteria accounts for 40 to 50 % of the lactate utilizers (Kim et al., 2000). The focus of Propionibacterium has been on propionate production rather than lactate fermentation despite its role as lactate utilizer. Propionate account for approximately 65 % of glucose release (Huntington, 2000) in growing ruminants and lactating cows and is reported to be quantitatively the most important single precursor of glucose synthesis, greatly affecting the release of hormones release and tissue distribution of nutrients (Nagaraja et al., 1997). In addition, propionate spares glucogenic amino acids minimizing the cost of maintenance of metabolisable protein (Van Soest, 1994).

Supplementation of a combination of Propionibacterium and *Lactobacillus* was developed to benefit from individual characteristics of microorganisms within the ruminal ecosystem. The inhibition of methane production in the rumen by *Lactobacillus* may improve animal performance through increased propionate production by Propionibacterium and improved energy efficiency (Krehbiel et al., 2011).

2.2.3. Bacterial Direct-Fed Microbials and the gut

2.2.3.1. Competition

Early research suggested that bacterial DFM could compete with pathogens for sites of adherence on the intestinal surface (Jones and Rutter, 1972). This was supported by the observation in piglets dosed with *L. lactis*, when homogenates of washed intestinal tissue had greater numbers of attached *Lactobacilli* and lower *E. coli* counts than control pigs (Muralidhara et al., 1977). *Lactobacillus acidophilus* 27SC was found compatible with the gastro-intestinal tract of young calves, confirming its adherence (Abu-Tarboush et al., 1996).

The adhesion of DFM seems to be mediated either by physicochemical factors, or by adhesive bacterial surface molecules and/or epithelial receptor molecules (Holzapfel et al., 1998). The ability of bacteria to adhere to epithelial cells depend on the polysaccharides forming the outer layer of the bacterial cell wall and the one forming layer on the intestinal cells (Fuller and Brooker, 1974).

2.2.3.2. Antibacterial effect.

Many species of lactobacilli has been shown to be antagonistic to pathogens such as *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Clostridium perfringens* (Gilliland and Speck, 1977). In piglets, supplementation with lactic acid decreased counts of coliforms (Ratcliffe et al., 1986). The mechanism by which lactobacilli seems to act include hydrogen peroxide produced (Gilliland and Speck, 1977) and reduction in pH (Fuller, 1977), preventing growth of many pathogens. A summary of published work and proposed mode of action of DFM used in ruminants studies (McAllister et al., 2011) is presented in table 2.2.

Table 2.2. Modes of action of Direct fed microbial used in ruminant studies (Adapted from McAllister et al., 2011)

Bacteria	Application	Mode of action
Lactic acid producers		
<i>Enterococcus faecium</i>	Calves, dairy cattle, Feedlot cattle, lamb	<ul style="list-style-type: none"> • Stimulation of lactic acid utilizers in calves • Competitive exclusion in dairy cattle
<i>Lactobacillus planterum</i>	Calves, dairy cattle, Feedlot cattle, lamb	
<i>Lactobacillus casei</i>	Dairy cattle, lamb	
<i>Lactobacillus acidophilus</i>	Calves, dairy cattle, Feedlot cattle, lamb	
Rumen bacteria		
<i>Megasphaera elsdenii</i>	Dairy cattle, Feedlot cattle, Lamb	<ul style="list-style-type: none"> • Increase propionate in dairy cattle • Moderation of pH in Feedlot cattle
<i>Prevotella bryantii</i>	Dairy cattle	
<i>Selomonas ruminantium</i>	Lamb	
Other		
<i>Propionibacterium</i>	Feedlot cattle	Increase propionate + Moderation of
<i>Propionibacterium jensenii</i>	Calves, dairy cattle	
<i>Propionibacterium acidopropionic</i>	Calves	
<i>Bifidobacterium spp</i>	Calves	Lower tract function in calves
<i>Bacillus spp</i>	Calves, dairy cattle,	Substrate utilization
<i>Eschericia coli</i>	Calves	Competitive exclusion
Yeast and fungi		
<i>Sacchoramyces cerevisae</i>	Calves, dairy cattle, Feedlot cattle, Lamb	<ul style="list-style-type: none"> • Rapid establishment of microbial consortia in new born calves • Improved fibre digestion in dairy
<i>Aspergillus oryzae</i>	Calves, Feedlot cattle	

2.2.3.3. Immune Response

Direct feed microbials promote intestinal health and overall well-being of the host through modulation of immunity (Isolauri et al., 2001). The animal immune system is capable of mounting immune responses against a variety of pathogens when encountered. Apart from its role in the digestion and absorption of nutrients, the GIT provides also its host a protective defence against a constant presence of antigens from food and microorganisms in the gut lumen. Besides epithelial cells, immune cells in the GIT consist of natural killer cells (macrophages, neutrophils, dendritic cells, and T and B lymphocytes). When orally infected by an antigen, immune cells are rapidly activated, which leads to enhanced phagocytosis as well as the production of humoral mediators (Zhang and Ghosh, 2001). Oral administration of Bacterial DFM such as *lactobacilli* generally resulted in an augmentation of innate immune

responses, as well as an elevated production of immunoglobulin A and a decreased immunoglobulin E production in animals (Erickson and Hubbard, 2000; Isolauri et al., 2001). However, influence of DFM on cytokine production and T and B cell responses show mixed results depending on the strain, dose, and duration of feeding DFM, as well as the type of tissues and cells analysed. *Lactobacillus rhamnosus* and *L. bulgaricus* strongly induced production of some promoting cytokines in peripheral blood mononuclear cells (Miettinen et al., 1998). *Lactobacillus johnsonii* showed a very low potential to induce pro-inflammatory responses, but rather favored the induction of TGF- β in an intestinal epithelial cell line (Haller et al., 2000). Some other species of probiotics are reported to be able of changing the immunomodulatory effects exerted by other species (Christensen et al., 2002). These reports indicate that bacterial DFM can provide protection against pathogens. Although several mechanisms are believed to be involved, the ability to adhere to and colonize the gastrointestinal tract appears to be the most likely important. More research are still needed to confirm the ability of DFM to act as immune modulators.

2.2.4. Direct-Fed Microbials supplementation of calves

Responses to feeding bacterial DFM have been variable in ruminant production systems, emphasizing the need for better understanding of mode of action. In ruminant production, effects of bacterial DFM have been studied most extensively in the pre-weaned dairy calf. Microbiological criteria considered as keys for DFM to be efficacious include safety, survival through regions of the gut, specificity to the host, and genetic stability (Holzapfel et al. 1998). The use of nonhost-specific species seemed be the reason of no response to bacterial DFM (Holzapfel et al. 1998).

Newman and Jacques (1995) reviewed data of studies of *Lactobacillus*, *Enterococcus*, *Streptococcus*, and *Bifidobacterium*. In general, the importance of bacterial DFM (primarily *Lactobacillus* species) fed to young and/or stressed calves has been to establish on maintaining “normal” intestinal microorganisms rather than as a production stimulant. Bacterial DFM have been reported to alter the balance of intestinal microorganisms, adhere to intestinal mucosa, preventing adherence of pathogen, influence gut permeability, and modulate immune function (Salimen et al., 1996). For dairy calves, rapid adaptation to solid feed by accelerating the establishment of ruminal and intestinal microorganisms and avoiding the establishment of enteropathogens, which often results in diarrhea, seems to be the primary goal. In the neonate

and in stressed calves, the microbial population is in transition and extremely sensitive; abrupt changes in diet or the environment can cause alterations in microbial populations in the gastrointestinal tract, which often leads to an increased incidence of diarrhea (Savage, 1977) and reduce lactobacillus in the gut (Sandine, 1979).

Performance of pre-weaned calves supplemented with bacterial DFM has been variable. Some studies reported no improvement in daily gain as a result of feeding *lactobacilli* (Ellinger et al., 1978; Abu-Tarboush, 1996), other reported improved performance (Bechman et al., 1977). The majority of studies indicate that the addition of DFM in calf feeding systems improves ADG, daily feed intake, and feed conversion. Supplementing *L. acidophilus* in milk replacer increased ADG in neonatal calves, with no effect on diarrhea occurrence (Cruywagen et al., 1995). In another study using Holstein calves, feeding nonviable *L. acidophilus* in milk showed increased starter intake with no effects on average daily gain (Higginbotham and Bath, 1993). When administered in milk replacer to pre-weaned calves from 7 days to 35 days of age, *Bifidobacterium pseudolongum* or *Lactobacillus acidophilus* improved body weight gain and feed intake. Additionally, feed conversion for treated calves was superior to that of non-treated calves (Abe et al., 1995). However, dietary supplementation of *Lactobacillus acidophilus*, *Aspergillus oryzae*, and *Bacillus subtilis* to Holstein calves had no effect on growth and feed conversion (Windschitl et al., 1991). Multi-species probiotics have been shown to decrease diarrhea occurrence in calves (Timmerman et al., 2005; Abe et al., 1995). Feeding *Lactobacillus* and *Streptococcus* to calves reduced the incidence of diarrhea (Abu-Tarboush et al., 1996) and the decreased incidence of diarrhea was associated with a consistently increased shedding of *Lactobacillus* (Jenny et al., 1991; Abu-Tarboush et al., 1996).

2.2.5. Identification of bacteria

Until recently, most of the methods of identification of bacteria in the GIT were based on various cultivation techniques. It was reported that those methods and phenotypic characterization have underestimated the diversity of bacterial and that the isolated and identified bacteria represent only approximately 20 % of species present in GIT (Amann *et al.*, 1995). The gapped BLAST program is also used to identify bacteria strains (Altschul *et al.*, 1997). The program is based on searching protein and DNA data base for sequence similarities and automatically combine statistically significant alignments produced by BLAST into a position-specific score matrix, and searching the database using this matrix. Currently preferred

molecular methods are those that allow more complete and rapid assessment of the biodiversity in this ecosystem. During the past decade, the identification based on the sequencing of genes coding ribosomal 16S rDNA has become a very important tool used for studying bacterial communities in animals (Kocherginskaya *et al.* 2001; Píknová *et al.* 2006; Loman *et al.* 2012).

2.3. Rumen development in calves

2.3.1. Rumen anatomy of the new born calf

The rumen of the neonatal calf is underdeveloped, representing approximately 30 % of the total gastro-intestinal capacity. The reticulum, rumen, and omasum are non-functional, and the rumen muscularization, and papillae growth are minimal (Heinrichs and Lesmeister, 2005). As the calf ages, the reticulum, rumen, and omasum size change (Figure 2.1). The rumen function and enzymatic activity is similar to a monogastric animal (Warner *et al.* 1956), and because of the non-existing metabolically activity, ruminal epithelial cells cannot convert butyrate to β -hydroxybutyrate (Heinrichs and Lesmeister, 2005).

The milk bypasses the rumen, passing through the esophageal groove direct to the omasum before passing to the abomasum (Wallace and Newbold, 2007). This allows the milk to be digested and absorbed (Lukas *et al.*, 2007). For the calf to become a ruminant, rumen development is necessary and will be affected by factors such as the quality and quantity of the feed consumed (Heinrichs and Lesmeister, 2005).

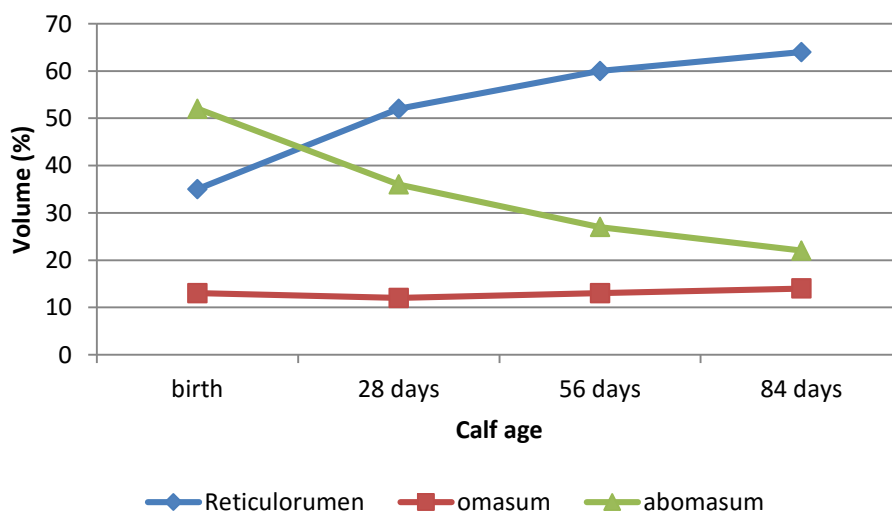


Figure 2.1. Composition of the rumen growth at various ages (Adapted from Church, 1976)

2.3.2. The developing rumen and microbial population

As the young calf start consuming solid feed, the microbial population increases and the rumen start developing (Dehority and Orpin, 1988). The development and extension of the calf rumen is encouraged by VFA, the end products of microbial fermentation (Warner et al., 1956). Total anaerobic bacterial counts increase considerably during the first 21 days of life (Beharka et al., 1998). The types of microbial population change with calf age (Beharka et al., 1998). Ruminal microbial population is reported to be also influenced by the physical form of the diet. Feeding ground diets is reported to increase amylolytic and reduce the amount of cellulolytic bacteria when compared to feeding unground diets (Beharka et al., 1998). Hespell (1981) summarized fermentative properties of rumen bacteria (Table 2.3).

2.3.3. Rumen epithelial metabolism

As the calf ages, the activity of the rumen epithelial metabolism increases and glucose absorbed in the small intestine remains the first energy substrate for ruminants. The importance of butyrate increases during rumen epithelial growth, while the importances of glucose and lactate oxidation decrease (Giesecke et al., 1979). The body uses beta-hydroxybutyrate (BHBA) as an energy source (Quigley et al., 1991) via the rumen epithelial cells that oxidize butyrate and converted it to BHBA through the rumen wall (Lesmeister and Heinrichs, 2004). Lane et al. (2000) indicated that the rumen epithelium ketogenic capacity to produce D-3-hydroxybutyrate from butyrate increases with age independently of solid feed intake, but the ability of the rumen mucosa to absorb volatile fatty acids increases with solid feed intake, but not with age (Sutton et al., 1963). Lower rumen pH increases the proportion of non-esterified fatty acids, increasing the rate of VFA absorption towards the blood stream (van Soest, 1994). Ketogenesis in the ruminal epithelium takes place in the mitochondria (Leighton et al. 1983). It can progress through 3-hydroxy-3-methylglutaryl- CoA (HMG-CoA) synthase and HMG-CoA lyase or via deacylation of acetoacetate-CoA catalyzed by succinyl- CoA transferase resulting in the production of acetoacetate. The final step in ruminal ketogenesis is the production of BHBA catalyzed by BHBA-dehydrogenase.

In the fed ruminant, butyrate is taken up from the rumen fluid into the reticulo-rumen mucosal epithelial cells, and converted to acetoacetate and BHBA because of the NADH: NAD ratio within the mitochondria (Heitmann et al. 1987). There are sufficient activities of ketogenic

enzymes present within the ruminal epithelium to account for the ketogenic capacity of the rumen (Baird et al. 1970, Bush and Milligan 1971, Leighton et al. 1983).

2.3.4. Rumen physical structure

The rumen physical development consists on rumen papillae growth, rumen weight and muscle. Lesmeister et al. (2004b) suggested papillae length and width, and rumen wall thickness as preferable indicators of rumen development. These parameters are significantly affected by diet composition and intake.

Table 2.3. Important bacterial species in cattle and sheep and their fermentative properties (Adapted from Hespell, 1981)

Species	Function*	Products¶
<i>Fibrobacter (Bacteroides) succinogenes</i>	C,A	F,A,S
<i>Ruminococcus albus</i>	C,X	F,A,E,H,C
<i>Ruminococcus flavefaciens</i>	C,X	F,A,S,H
<i>Butyrivibrio fibrisolvens</i>	C,X,PR	F,A,L,B,E,H,C
<i>Clostridium lochheadii</i>	C,PR	F,A,B,E,H,C
<i>Streptococcus bovis</i>	A,S,SS,PR	L,A,F
<i>Ruminobacter (Bacteroides) amylophilus</i>	A,P,PR	F,A,S
<i>Prevotella (Bacteroides) ruminicola</i>	A,X,P,PR	F,A,P,S
<i>Succinimonas amyolytica</i>	A,D	A,S
<i>Selenomonas ruminantium</i>	A,SS,GU,LU,PR	A,L,P,H,C
<i>Lachnospira multiparus</i>	P,PR,A	F,A,E,L,H,C
<i>Succinivibrio dextrinosolvens</i>	P,D	F,A,L,S
<i>Methanobrevibacter ruminantium</i>	M,HU	M
<i>Methanosarcina barkeri</i>	M,HU	MC
<i>Treponema bryantii</i>	P,SS	F,A,L,S,E
<i>Megasphaera elsdenii</i>	SS,LU	A,P,B,V,CP,H,C
<i>Lactobacillus sp.</i>	SS	L
<i>Anaerovibrio lipolytica</i>	L,GU	A,P,S
<i>Eubacterium ruminantium</i>	SS	F,A,B,C
<i>Oxalobacter formigenes</i>	O	F,C
<i>Wolinella succinogenes</i>	HU	S,C

* C = cellulolytic; X = xylanolytic; A = amylolytic; D = dextrinolytic; P = pectinoiytic; PR = proteolytic; L = lipolytic; M = methanogenic; GU = glycerol-utilizing; LU = lactate-utilizing; SS = major soluble sugar fermenter, HU = hydrogen utilizer; O = oxalate-degrading.

¶ F = formate; A = acetate; E = ethanol; P = propionate; L = lactate; B = butyrate; S = succinate; V = valerate; CP = caproate; H = hydrogen; C = carbon dioxide; M = methane.

Therefore, early intake of solid feed stimulates rumen development. Papillae denseness measurements was found to vary across different rumen sections in contrast to papillae length and width that were reported to be similar among rumen sections (Hill et al., 2005).

2.3.5. The developing rumen and fermentation

2.3.5.1. Volatile fatty acids

Volatile fatty acids or short-chain fatty acids (SCFA) are low-molecular mass carboxylic acids, which presence in a sample matrix is often indicative of bacterial activity (Siedlecka et al., 2008). Volatile fatty acids originate from anaerobic biodegradation of organic matter and since

they are involved in different processes in the development of the rumen, their determination has become of increasing interest (Siedlecka et al., 2008).

In order for the rumen to develop, sufficient concentrations of rumen VFA, especially butyrate and, to a lesser degree, propionate, is required (Coverdale et al., 2004). The necessary ruminal butyrate concentration required to stimulate rumen papillae development is provided by fermentation of calf concentrate. Many previous studies have demonstrated increased rumen development with butyric and propionic acids (Lane and Jesse, 1997). The effect of butyric acid and propionic on rumen papillae development is reported to be most likely associated with the rate at which they are metabolized by mucosal cells. During absorption, over 90 % of butyric acid and approximately 50 % of propionic acid is metabolized and oxidized to ketone bodies (Britton and Krehbill, 1993).

Type and physical form of diet intake influence rumen physical development. Feeding only milk to calves does not stimulate papillae development, but the consumption of solid feed, and the resulting increase in rumen VFA concentrations stimulated rumen morphological development (Assane and Dardillat, 1994; Lane et al., 2000). In the same manner, reducing age of calf at weaning, stimulate starter feed consumption and papillae development (Cozzi et al., 2002; Klein et al., 1987). Hay stimulates rumen volume and weight of rumen content, while concentrate diets increases the weight of reticulo-rumen tissue (Stobo et al., 1966). The increase in weight of reticulo-rumen tissue in calves fed a high-concentrate diet was attributed to a greater papillae length rather than a growth of the muscular rumen wall (Stobo et al., 1966). Similarly, calves fed a concentrate diet compared with a concentrate plus alfalfa diet presented rumen papillae with protrusions, that increase surface for absorption, but the presence of protuberances was not related to rumen hyperkeratosis. In contrast, longer papillae without protrusion were observed with a concentrate-alfalfa hay diet compared with a concentrate diet (Zitnan et al., 1998). In general, high-concentrate diets stimulate rumen mucosa development by increasing papillae length and surface, in contrast to diets containing some forage that increased the proportion of muscle of rumen wall, and presented less vacuolation of stratum granulosum and thickening of the stratum corneum suggesting a favored metabolic uptake (Nocek et al., 1984). Hence, butyrate and propionate are the main fermentation products from a concentrate diet, which are more responsible for stimulating papillae growth (Warner, 1991).

The effect of grinding the calf starter did not affect the ruminal VFA concentration and rumen wall thickness (Beharka et al., 1998). However, calves fed an unground calf starter had longer rumen papillae in the dorsal sac in contrast to calves fed a ground calf starter that showed evidence of branched papillae, which were not accompanied by an increase of rumen mucosa parakeratosis (Beharka et al., 1998). Corn processing slightly influenced rumen physical development in a study reported by Lesmeister and Heinrichs (2004).

2.3.5.2. Circulating beta-hydroxybutyrate

Apart from decreased glucose oxidation and increased VFA oxidation, which occur in the rumen epithelium in concert with morphological development, increased production of ketone bodies from butyrate occurs (Baldwin et al., 1992; Giesecke et al., 1979) as part of metabolic changes. Beta-hydroxybutyrate is a relatively simple chemical structure and is synthesized from absorbed butyrate in the rumen epithelium of ruminants and by the ketogenesis of the liver cells in the conversion of long-chain fatty acids from fat mobilization. As is the case with acetoacetate, BHBA is freely distributed and transported in the blood, and seem interconvertible in various tissues and seems to contribute to the overall energy consumption in the body to a non-negligible degree (Steel et al., 2009).

In adult ruminants, BHBA is often produced when energy is limited and the body mobilizes fat. This condition is called ketosis and may lead to clinical problems (Enjalbert *et al.*, 2001). In young animals, when the calf begins to consume calf starter the physical development of the stomach is associated with changes in blood BHBA (Quigley et al., 1991). Beta-hydroxybutyrate is then used as an indicator of the development of the rumen, and is produced when rumen bacteria ferment carbohydrate in the rumen to butyric acid. The development of rumen mucosa is stimulated by the presence of butyrate and/or propionate (Greenwood et al., 1997; Beharka et al., 1998; Baldwin et al., 2004), which is most likely associated with the rate at which these acids are metabolized by mucosal cells. As mentioned earlier, around 50 % of propionate is metabolized during absorption and over 90 % of butyrate is oxidized to ketone bodies (Britton and Krehbill, 1993). Butyrate is then absorbed by the rumen epithelial cells and converted to BHBA (Quigley et al., 1991). Blood BHBA was found to increase in calves offered calf starter from 140 mM/l at week 1 to about 800 mM at week 10 (Figure 2.2). The increase was found to be closely related to the increase in starter intake (Quigley et al., 1991).

Direct addition of butyric acid or its sodium salt to the calf starter was considered to be used to accelerate rumen development, which is reflected in plasma BHBA (Hill et al., 2007b). Addition of sodium butyrate to milk replacer positively affected BW and ADG. Inclusion of sodium butyrate into starter feed increased starter diet intake from d 15 to 21, increased ($P<0.05$) plasma

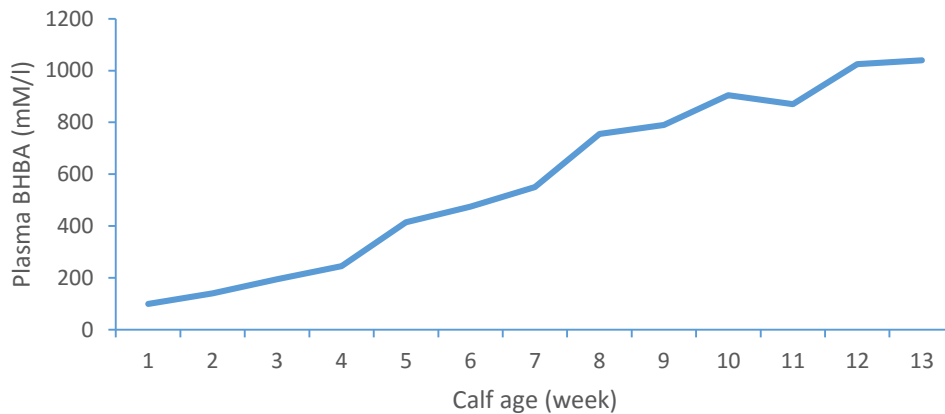


Figure 2.2. Effects of age on plasma BHBA of calves fed starter feed from day 4 for weaning at 56 d (Adapted from Quigley et al. 1991)

glucagon-like peptide-2 (GLP-2) concentration on d 7 of the trial when compared with the concentration on d 0. Addition of sodium butyrate into both milk replacer and starter feed increased reticulorumen weight and papillae length and width. These increase correlated with increased level of plasma BHBA.

The enzymatic conversion of acetoacetate to BHBA is assessed as a decrease in absorbance spectrophotometry at 340 nm, while the reverse process, the oxidation of BHBA to acetoacetate, is assayed as an increase in absorbance (Young and Reynold, 1966). Analyses of BHBA in blood plasma using the traditional enzymatic spectrophotometric method are susceptible to background absorbance in the sample, especially if blood samples are hemolysed (Jacobs et al., 1992; Duffield et al., 1998). Insertion of a proper sample blank or use of efficient second wavelength detection in the analyses may minimize this problem. The fluorometric determination has been suggest as more precise, with the advantage that liquid samples do not need pretreatment to obtain a transparent status. In addition, the developed fluorophore is easily read in nontransparent liquids (Larsen and Nielsen, 2005). According to the latter, the fluorometric determination appears to be a more reliable method for analysis of plasma. The assay is easily automated to permit the handling of large numbers of samples.

2.3.5.3. Ruminant Ammonia

Ammonia nitrogen ($\text{NH}_3\text{-N}$) is a major protein metabolite in the rumen and the principal end product of microbial protein degradation of feedstuffs. It is also the nitrogen form required for most strains of rumen bacteria and has been used as an indicator of the efficiency of microbe protein degradation and of non-protein nitrogen utilization (Broderick and Kang, 1980). A decrease in $\text{NH}_3\text{-N}$ concentration in the rumen is attributed to ruminal microbial proliferation, due to the increase of microbial use of available $\text{NH}_3\text{-N}$ (Crocker et al, 1998). Beharka at al. (1998) reported low $\text{NH}_3\text{-N}$ concentrations in Holstein bull calves, fed, in addition to milk, either a finely ground compared to unground diet consisting of chopped hay and rolled grain. Lesmeister and Heinrichs (2004) observed a similar pattern for Holstein calves fed starter containing dried rolled maize for 42 days compared to calves fed starter containing steam-flacked maize. Their results indicated an increased incorporation of $\text{NH}_3\text{-N}$ into microbial protein.

2.3.5.4. 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 NH_3 , with lactate being the primary acid and bicarbonate and phosphate acting as major buffers.

2.3.6. Feedstuffs and rumen development.

Dietary change has direct impact on rumen development, involving physical size, wall thickness and papillae formation (Heinrichs and Lesmeister, 2005). Inoculation and establishment of the anaerobic microbial ecosystem of the rumen, initiation of solid feed intake, fermentation and absorption mechanisms must take place for the rumen to develop (Williams and Frost, 1992; Baldwin et al., 2004). In addition, some changes must occur at the hepatic and intestinal levels in order for the calves to make use of end-products of fermentation (Baldwin et al., 2004; Drackley, 2008). Rumen development and microbe proliferation is depending on intake and type of feed. The rumen of calves fed only milk or milk replacer develop very little, while consumption of solid feeds stimulates rumen microbial growth and VFA production (Heinrichs and Lesmeister, 2005). Calves rely on the nutrients obtained from milk during the first few weeks of life, which limits rumen development (Heinrichs and Lesmeister, 2005).

When calf starter intake is limited, metabolic activity and volatile acids production and absorption is minimal (Heinrichs and Lesmeister, 2005), limiting rumen epithelial growth and muscularisation. As the calf grows, the rumen size will still increase regardless of the development of the rumen (Vazquez-Anon et al., 1993), resulting at weaning in an unhealthy calf with limited growth due to its inability to digest grain and/or forages (Heinrichs and Lesmeister, 2000).

Concentrate feeds contribute to a high rate of rumen development, and their intake increases microbial proliferation and VFA production and influences rumen epithelial development and wall vascularization (Heinrichs and Lesmeister, 2000). 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 increase the surface area of the rumen wall and allow for greater absorption (Heinrichs and Lesmeister,

2005). Increased intake of concentrate decreases rumen pH, while forage intake maintains a higher ruminal pH due to larger particle size without promoting rumen epithelial development (Heinrichs and Lesmeister, 2005). However, forages promote rumen muscle development, and stimulate rumination and saliva production in the rumen (Coverdale et al., 2004). The influence of calf age on ruminal VFA is illustrated in Figure 2.3 and 2.4.

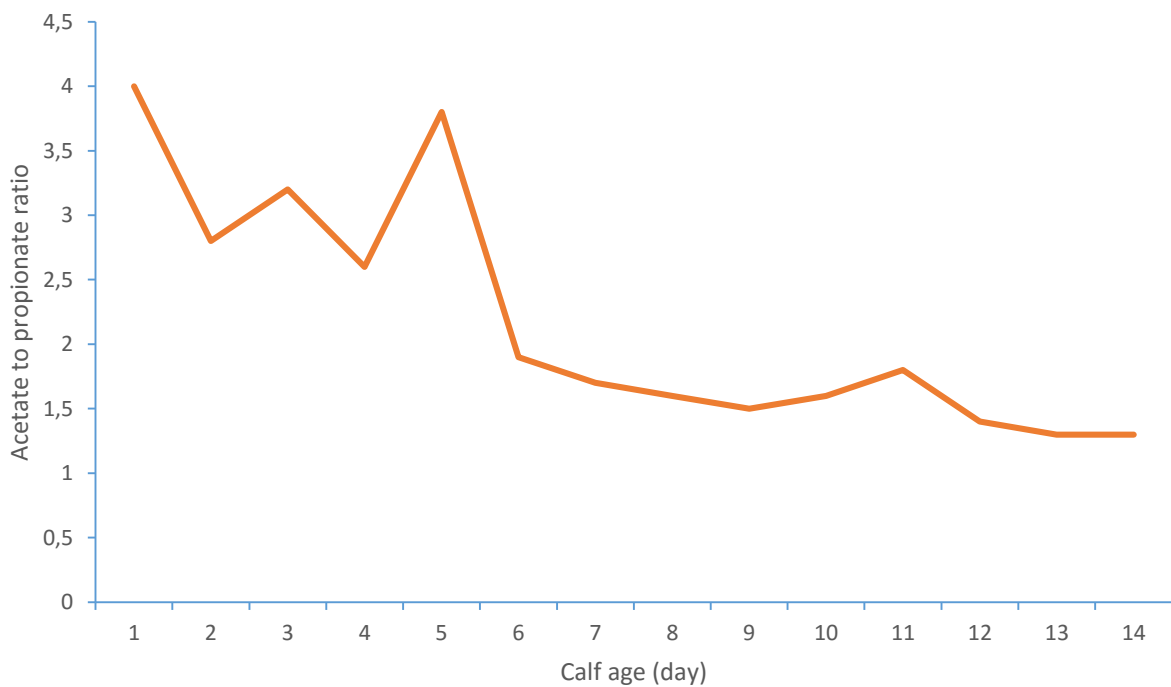


Figure 2.3. Effect of calf age on ruminal acetate to propionate ratio (Adapted from Rey et. al, 2012).

According to Heinrichs and Lesmeister (2005), factors that causes development of the rumen include: 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.

At birth, no fermentative activity occurs in the rumen of a calf due to the absence of VFA, xylanase and amylase activities and a weak capacity of enzymatic degradation at that age (Rey et al., 2012). The latter observed an increase in total VFA concentration from d 2 to 12 of age to reach a plateau (84.4 mM). Other reports (Anderson et al., 1987; Beharka et al., 1991) also indicated that VFA concentrations increases from week 1 to 1 month of age to reach a stable value. Rey et al. (2012) conclude that the establishment of the papillae and the ruminal mucosa consecutive to the appearance of fermentation end products improves VFA absorption. Between d 10 and 30 after birth, the ruminal pH decreases with a time pattern that was opposite

to that of total VFA content due to the progressive intake of solid feed, providing substrates to the ruminal microbiota (Rey et al., 2012).

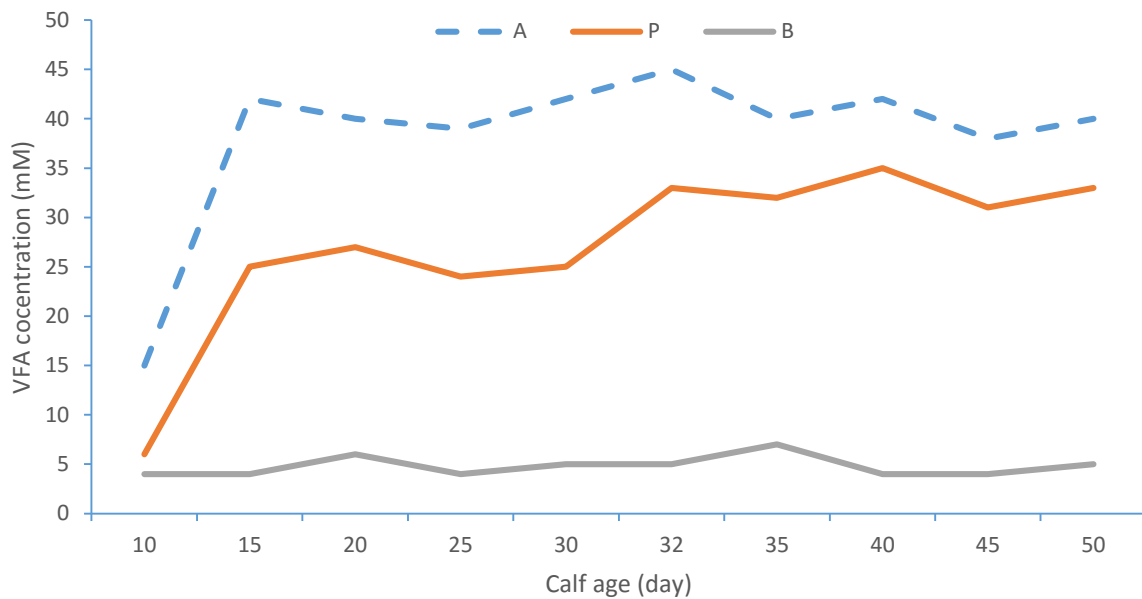


Figure 2.4. Effect of calf age on ruminal proportion of VFA's (Adapted from Rey et. al, 2012).

Early consumption of concentrate feed is essential for promoting rumen development. At birth, the calf's rumen is sterile, and progressively concentration of bacteria establishes, mostly aerobic. As the calf consumes dry feed, the bacteria numbers and type change predominantly to anaerobes (Beharka et al., 1998). Water offered earlier to calves, results in increased daily weight gain, concentrate intake, and reduces scours (Heinrichs and Lesmeister, 2005). Given these benefits of high concentrate intakes, several studies have been undertaken to address the need of limiting milk intake in order to stimulate starter intake. Little work has specifically addressed differences in ruminal morphology in relation to milk intake.

Kristensen et al. (2007) fed a barley-based starter concentrate with varying amounts of milk to calves and showed that calves supplied a daily allotment of 3.1 versus 8.3 L of milk had heavier forestomachs (as % of BW), but found that, regardless of milk allowance, the ruminal environment of young calves was characterized by a low ruminal pH and high VFA concentration. These authors attributed the heavier forestomachs in the restricted-fed calves to greater solid feed consumption when compared to the calves receiving more milk. Roth et al. (2009) found that the length of papillae in the atrium or ventral ruminal sac was not affected

by milk allowance or by the resulting variation in solid feed consumption in calves. Khan et al. (2007b) found that providing calves a large amounts of milk early in life and then reducing intakes before weaning (step-down method) caused a surge in solid feed consumption. They also reported a metabolically more developed and heavier forestomach in calves fed milk through the step-down method compared with those fed restricted quantities of milk. Roth et al. (2009) reported similar starter intakes and rumen development in calves weaned using concentrate-dependent (milk supply was reduced once calves consumed 2 kg/d of starter for 4 consecutive days) or conventional weaning methods (abruptly removing all milk at 12 wk of age). The lack of treatment differences in the latter study may be explained by the relatively low amount of milk offered to both groups of calves.

2.4. *Megasphaera elsdenii*

Isolated from the rumen of cattle in 1953, *M. elsdenii* was first called organism LC before being named in 1956, *P. elsdenii* (Elsden *et al.*, 1956). It was later on observed that *P. elsdenii* was different from other members of the genus *Peptostreptococcus* (Rogosa *et al.*, 1971), and was reclassified and called *M. elsdenii* (Rogosa, 1971). *Megasphaera elsdenii*'s substrate preferences are, in order; glucose, maltose, lactate and sucrose (Russell and Baldwin, 1978). *Megasphaera elsdenii* is considered to be the predominant lactate-utilizing bacterium capable of fermenting up to 97% of rumen lactate (Counotte *et al.*, 1981). In addition to lactate fermentation, *M. elsdenii* plays a major role in volatile fatty acids production and converts glucose, maltose, lactate and sucrose into acetic, butyric and finally *n* hexanoic acid *via* a series of condensations of acetyl-CoA derived from the metabolism of pyruvate to generate the extra reducing equivalents as NADH₂ (Pacaud *et al.*, 1985; Roddick and Britz, 1986).

Megasphaera elsdenii inoculation has modified ruminal fermentation and prevented the accumulation of lactate during the transition from low- to high- concentrate diets in both *in vitro* and *in vivo* studies (Kung and Hession, 1995). The latter also reported that the pH of cultures supplemented with *M. elsdenii* (106 cfu/ mL of culture fluid) decreased below 5.5 at 4 h and remained at approximately 5.3 (24-h culture), whereas the control decreased to pH 4.8. Lactate concentration peaked at more than 40 mM in control after 8 h and remained fairly constant thereafter, but in the *M. elsdenii* treatment, it was less than 5 mM through incubation. Total VFA concentration of cultures treated with *M. elsdenii* was more than twice that of

control (131.4 vs. 63.3 mM). Acetate concentration, however was not different after 2 h ($P < 0.05$). The concentration of propionate, butyrate, valerate, isobutyrate, and isovalerate for control and *M. elsdenii* inoculation at 6 h were 38:47, 2:35, 1:15, 1:11, and 1:2 (mM, control:*M. elsdenii*), respectively. Therefore, most differences in VFA concentration between treatments resulted from increased butyrate, valerate, and branched-chain fatty acids. Greening et al. (1991) reported that inoculation with *M. elsdenii* decreased minimal pH and lactate concentration in acidosis induced beef cattle. Minimal pH for control, inoculation prior to acidosis induction, and 0 h, or 2 h after acidosis induction were 4.65, 4.73, 5.51 and 5.26 and maximal lactate concentrations were 124, 121, 49.9, and 45.9 mM, respectively. Accumulated total VFA were 472, 507, 910, and 870 mM for respective treatments. Robinson et al. (1992) reported the effects of inoculation with *M. elsdenii* on feed intake, ruminal pH, osmolarity, lactate, and VFA concentration in acute acidosis-induced steers fed a 90% concentrate diet. In that study, the interaction between inoculation and day of diet switch moderated pH, lactate, VFA, and feed intake significantly. The DMI of steers inoculated with *M. elsdenii* was increased by 24%.

Selected from strains isolated from rumens of concentrate-adapted fistulated dairy cows and slaughtered feedlot cattle, a specific strain of *M. elsdenii* (NCIMB 41125) has shown to be the most promising and thriving according to a set selection criteria (Horn *et al.*, 2009a). These included: high growth rate, ability to metabolize lactate and produce fermentation end products at pH levels well below 5.5 and its ability to replicate at a measurable μ_{max} at pH = 4.5 compared to other strains of *M. elsdenii* (minimal activity and replication ability at a pH range of 4.5 - 5.5). In addition, the organism is able to convert lactate to (primarily) acetate and continues to do so at low pH levels. The strain NCIMB 41125 was also reported to be resistant to ionophores, most in-feed anthelmintics and antibiotics (Apajalathi *et al.*, 2008; Rinttilä *et al.*, 2009). Hino and Kuroda (1993) suggest possible metabolic pathways from glucose, lactate, and acrylate to main VFA with *M. elsdenii* (Figure 2.5).

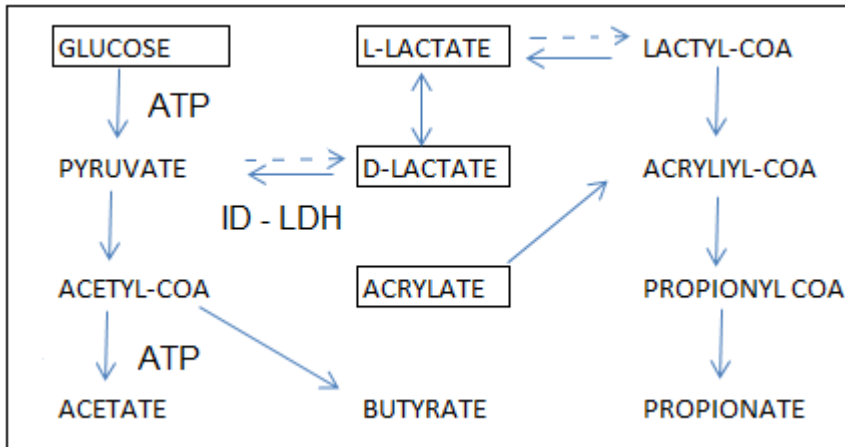


Figure 2.5. Metabolic pathways from glucose, lactate, and acrylate to main VFA with *M. elsdenii* (Adapted from Hino and Kuroda, 1993).

Megasphaera elsdenii (NCIMB 41125) was characterized as follows in terms of its ability to prevent ruminal acidosis:

- It displays a high growth rate of up to 0.938/h (Horn *et al.*, 2009b)
- Its biomass accumulation provides more tolerance to environmental stressors such as oxygen exposure which makes it highly robust for commercial use (Meissner *et al.*, 2010).
- It is able to metabolize lactate and produce fermentation end products at pH levels well below 5.5 and it is able to replicate at a measurable μ_{max} at pH = 4.5 (Horn *et al.*, 2009b).
- It has the ability to convert lactate to (primarily) acetate and continues to do so at low pH levels (Horn *et al.*, 2009b).
- The isolate is unaffected by ionophores, by most in-feed anthelmintics and antibiotics (Apajalathi *et al.*, 2008; Rinttilä *et al.*, 2009).

2.4.1. Reported benefits associated with *M. elsdenii* in ruminant feeding

In a previous study reported by Henning *et al.* (2010b), sheep were fed roughage and then supplemented without adaptation 1000 g of maize followed by 300 g of maltose through the rumen cannula followed by administration one hour later of strain NCIBM 41125 into the rumen. The latter reported that ruminal pH remained above 5.5 from 8 h to 24 h post-administration ($P < 0.001$), while in the control treatment it remained below 5 for the entire

period. Lactic acid in the control treatment increased progressively to >55 mM at 10 h, whereas it remained below 10 mM in the strain 41125 administered treatment ($P < 0.05$).

Megasphaera elsdenii (strain NCIMB 41125) was found to be efficient against lactic acid accumulation in the rumen of cattle (Kettunen et al., 2008; McDaniel et al., 2008; Henning et al., 2010a). In a 100-day feeding trial using steers, Henning et al. (2009) reported that *M. elsdenii* NCIMB 41125 improved ADG during the immediate post-adaptation phase. In addition, morbidity levels were lower on the low roughage diet. In two other studies using lambs, the objectives of Henning et al. (2010b) were to first evaluate feed intake and ruminal acidosis when the diet is rapidly transitioned from forage to concentrate and secondly to investigate whether drenched, *M. elsdenii* (strain NCIMB 41125) can be traced and measured in the rumen and investigate whether a viable lactate utilizing population can be established during transition. In the first experiment, drenched lambs consumed more concentrate but less forage. Total feed intake was also improved with less variation in DMI. No difference in ADG and dressing % was observed. Rumen pH declined less in drenched lambs with less lactic acid during days +2 and +3. In the second experiment, feed intake and ADG were higher in drenched animals. The PCR results reflected higher concentrations of the strain during days +2 and +3 ($P = 0.06$) in treated lambs.

2.5. Conclusion

From this literature review it is clear that the early development of the rumen in the liquid fed calf is highly dependent on the rapid establishment of a rumen population and the production of sufficient quantities of specific volatile fatty acids, such as butyrate and propionate. This is applicable to both conventional and accelerated growth calf feeding systems, although there are limited comparisons in rumen development of calves between those systems.

A number of studies in ruminants have shown that the organism *M. elsdenii* is highly effective in modifying rumen fermentation patterns and preventing the accumulation of lactate during the transition from low to high concentrate diets, both *in vitro* and *in vivo*. This transition requires significant changes in the growth and development of the rumen papillae. Likewise, the liquid fed calf also needs significant changes in the growth and development of rumen papillae. No studies have been conducted on the use of *M. elsdenii* in the potential development of the rumen in the young calf, although theoretically it could have great potential and should

therefore be thoroughly investigated. The objectives and hypothesis of this investigation are discussed in chapter three, followed by a series of research studies reported in Chapters 4 to 7.

CHAPTER 3

OBJECTIVES

This thesis was conceived from:

1. Field work observations at a number of dairy farms that indicated improved performance in dairy calves supplemented with *Megasphaera elsdenii* (F. Hagg, personal communication, frans@alliednutrition.com).
2. Published research suggesting that:
 - *Megasphaera elsdenii* plays a major role in production of branched-chain volatile fatty acids in the rumen, and that its primary function is to convert lactic acid to propionate and butyrate and to convert glucose to butyrate (Marounek et al., 1989).
 - Supplementation with *Megasphaera elsdenii* resulted in butyrate as the main product of fermentation in a continuous culture with the ruminal bacterium using lactate as carbon source (Cruz et al., 2001).
 - Butyric acid provides energy for thickening of the rumen wall, formation of papillae, increasing capillary development (Weigand et al., 1975) and solid feed intake (Coverdale et al., 2004).
 - *Megasphaera elsdenii* NCIMB 41125 has the ability to continue the conversion of lactate to butyrate and/or propionate at low pH levels (Horn et al., 2009b).
 - *Megasphaera elsdenii* NCIMB 41125 shifted the fermentation towards butyric and valeric acid in feedlot cattle at still lower pH's (Henning et al., 2010a)

The primary objective of this work was to evaluate performance of pre-weaned Holstein calf when supplemented with *Megasphaera elsdenii* NCIMB 41125. The specific objectives were:

1. To determine total bacteria, *M. elsdenii* and *M. elsdenii* NCIMB 41125 counts after an oral dose of *M. elsdenii* NCIMB 41125.
2. To evaluate the effects of an oral dose of *M. elsdenii* NCIMB 41125 on the presence of VFA's in the rumen, colon and rectum of pre-weaned Holstein calves.
3. To determine the effects of an oral dose of *M. elsdenii* NCIMB 41125 on blood metabolites in calves.

4. To determine the effect of oral dose and time of dosing of *M. elsdenii* NCIMB 41125 on intake, growth, and diarrhea occurrence in pre-weaned Holstein calves in a conventional growth system.
5. To evaluate the influence of an oral dose of *M. elsdenii* NCIMB 41125 on pre- and post-weaning feed intake and growth of Holstein calves in an accelerated growth system.
6. To determine the effects of an oral dose of *M. elsdenii* NCIMB 41125 on serum beta-hydroxybutyric acid and papillae development.

To achieve these objectives, four studies were conducted:

Study 1: Effect of an oral dose of *M. elsdenii* 41125 on lower digestive tract bacteria counts, rumen fermentation and blood metabolites in Holstein calves

Study 2: Effects of dosing *M. elsdenii* NCIMB 41125 on feed intake, growth and some health parameters in pre-weaned Holstein calves in a conventional calf rearing system.

Study 3: Effects of dosing *M. elsdenii* NCIMB 41125 supplementation on growth and intake of neonatal Holstein calves having free access to milk during the feeding period (accelerated growth system).

Study 4: Rumen development, ruminal fermentation and blood beta-hydroxybutyrate synthesis in pre-weaned dairy calves dosed with *M. elsdenii* NCIMB 41125.

All these protocols were approved by the Animal Use and Care of Ethics committee of the University of Pretoria in accordance with the Guide for the Care and Use of Animal in Agriculture Research and Teaching (2010).

CHAPTER 4

EFFECT OF AN ORAL DOSE OF *MEGASPHAERA ELSDENII* NCIMB 41125 ON LOWER DIGESTIVE TRACT BACTERIA COUNTS, RUMEN FERMENTATION AND BLOOD METABOLITES IN HOLSTEIN CALVES

4.1. Introduction

More natural alternatives to antimicrobial feed additives that enhance health and performance of young calves are continuously being evaluated to replace antimicrobial additives (Heinrichs et al., 2003; Hong et al., 2005; Krehbiel et al., 2003 and Seo et al., 2010). Rumen development is an important factor determining early solid feed intake and performance in calves and lambs (Gorka et al., 2009). Improving rumen development, thereby increasing the rate of starter intake, is vital to the calf for transitioning from a pre-ruminant to a functioning ruminant (Coverdale et al., 2004). In addition, early rumen development and growth may impact on the lifetime performance of the calf (Anderson et al., 1987).

Butyrate and propionate are the primary VFA's responsible for rumen epithelial and papillae development in the pre-weaned ruminant (Coverdale et al., 2004). *Megasphaera elsdenii* has proven to play a major role in production of branched-chain VFA in the rumen (Allison, 1978; Wallace 1986; Stewart and Bryant. 1988) and may favour propionic acid as end product, but sometimes butyric acid at the expense of propionic acid (Henning et al., 2010a). It might be logically considered that DFM's may readily integrate, establish and persist into the microbial community as they are being introduced into the environment from which they were derived. However, reports have shown that cultured rumen bacteria sometimes fail to persist in the rumen (Krause et al., 2001).

Before conducting any trials, investigating the effect of *M. elsdenii* on the growth performance and health of pre-weaned calves, it is of important to first establish whether *M. elsdenii* would colonize and survive in the rumen and digestive tract of calves and how it affects volatile fatty acid production. This first study therefore could be regarded as a pilot study that would enable us to determine whether to continue or not with growth trials.

The primary objective of this pilot study was to determine total bacteria, *M. elsdenii* and *M. elsdenii* NCIMB 41125 counts in the rumen and colon digesta, and rectum fecal material of young Holstein calves after an oral dose of *M. elsdenii* NCIMB 41125 (10^8 CFU/mL), to

ensure that the bacteria would survive the acidity in the lower tract. The secondary objectives were:

- To evaluate the effects of an oral dose of *M. elsdenii* NCIMB 41125 on short chain volatile fatty acids in the rumen, colon and rectum of pre-weaned Holstein calves.
- To determine the effects of an oral dose of *M. elsdenii* NCIMB 41125 on blood gases in young calves.

4.2. Materials and methods

4.2.1. Animals and treatments

The experiment was carried out at the calf unit of the Dairy Research Unit at the Agricultural Research Council-Animal Production Institute, in Pretoria, South Africa (ARC-Animal production Institute, Old Olifantsfontein road, Irene, 0062), and was approved by the Animal ethics committee (APIEC 12/012).

Calves were housed in individual pens (with rubber mats) that prevented nose-to-nose contact between calves and prevent cross contamination of bacteria between animals.

Twenty four male Holstein calves were blocked on the basis of order of birth and used in a complete randomized block design with 2 treatments in a 2x3 factorial arrangement. Calves were either dosed with *M. elsdenii* NCIMB 41125 (Me) or not. The calves receiving *M. elsdenii* NCIMB were dosed with 50 ml containing 10^8 CFU/mL (Figure 4.1) and the control calves were not dosed. Within each of the two treatments groups, calves were divided into three sub-treatment groups (Not dosed: 7 d, 14 d and 21 d vs dosed: Me 7 d, Me14 and Me21 d). Each groups contained 4 calves within which two calves were euthanized at 24 h and two calves at 72 h. Each individual animal was regarded as an experimental unit unless otherwise stated and entered the trial at birth until euthanized at either 24 or 72 h after dosing time as is illustrated in Table 4.2. Therefore the effective experimental periods were: 8 or 10 days for calves dosed on d 7; 15 or 17 days for calves dosed on d14; 22 or 24 days for calves dosed on d 21.



Figure 4.1. Dosing of calves.

Calves were observed daily for possible health problems. After receiving colostrum for 3 consecutive days after birth, calves were fed whole milk (Table 4.3) and had free access to a commercial calf starter pellet (Table 4.1) and fresh water. All calves were euthanized within the stipulated times.

Table 4.1. Chemical composition of commercial calf starter¹ fed to calves not dosed or dosed with *M. elsdenii* NCIMB 41125

Chemical analysis	
DM, g/kg	885
CP, g/kg DM	181
Fat, g/kg DM	35
NDF, g/kg DM	219
ME, MJ/kg of DM ²	14.3
Ca, g/kg	8.1
P, g/kg	5.1

¹Contained a premix that supplied per kg: 15000 IU Vitamin A., 3000 IU Vitamin D3, 30 mg Vitamin E, 125 mg niacin, 50 mg Mn, 50 mg Fe, 50 mg Zn, 10 mg Cu, 0.8 mg I, 0.15 mg Co, 0.15 mg Se, 180 mg P, and 50 mg antioxidant.

²Metabolizable energy calculated according to NRC (2001).

The dry matter, crude protein, and fat contents were determined according to the standard AOAC procedures (2000). Calcium (Ca), potassium (K) and magnesium (Mg) were determined according to Giron, (1973) using a Perkin Elmer Atomic Spectrophotometer. Phosphorus (P) was assayed according to AOAC (2000) procedure 965.17. The NDF was analyzed using the methods of VanSoest et al. (1991) with an ANKOM fiber analyzer.

Table 4.2. Experimental layout of the study involving 24 calves dosed or not with *M. elsdenii* NCIMB 41125.

Treatments	Calf age (days)	Calves number	Samples	Euthanize time after dosing (hours)	n
Control (Not dosed)	7	2	2	24	4
		2	2	72	4
	14	2	2	24	4
		2	2	72	4
	21	2	2	24	4
		2	2	72	4
Oral dose of <i>M. elsdenii</i> NCIMB 41125	7	2	2	24	4
		2	2	72	4
	14	2	2	24	4
		2	2	72	4
	21	2	2	24	4
		2	2	72	4

Table 4.3. Milk¹ feeding regime of calves dosed or not with *M. elsdenii* NCIMB 41125

		Morning	Afternoon
Day 1-day 3	Colostrum	2 litres	2 litres
Day 4 to day 7	Milk	2 litres	2 litres
Day 8 to day 14	Milk	3 litres	3 litres
Day 15 to day 35	Milk	2 litres	2 litres
From Day 36 to weaning	Milk	2 litres	-

¹ Average milk composition: CP: 3.23 %, fat: 3.47 %, lactose: 4.69 %

4.2.2. Sampling and analyses

4.2.2.1. Fecal, digesta and blood samples

Fecal grab samples were taken from each calf dosed on d 14 in duplicate at -24 h and thereafter +24 h or +72 h relative to dosing *M. elsdenii* NCIMB 41125 for calves euthanized at +24 and +72 h respectively. Fecal samples were stored at -60°C until analysis. Immediately after euthanizing the calves at +24 h or +72 h, the digestive tract was harvested from all calves, and rumen and colon digesta samples were collected, and stored at -60°C until VFA determination and DNA extraction for determination of total bacteria, *M. elsdenii* and *M. elsdenii* NCIMB 41125 count using the 16s RNA PCR probe technique (Atlas and Bej, 1990). Jugular blood samples were taken from each calf at -2 h and +24 h or +72 h relative to dosing *M. elsdenii* NCIMB 41125 for calves euthanized at 24 and 72 h respectively. Blood was also collected using a 60 ml syringe and immediately analyzed for pH, using a portable pH meter. Additional blood samples were collected in a blood-gas-syringe, filled with heparin, from the artery on the inside of the hind leg for blood gas and blood lactate analysis. One drop of blood samples was used to analyse the lactic acid level in the blood, using an Accutrend Plus Instrument System (Roche Products (Pty) Ltd, Isando, South Africa) immediately after sampling. The rest of the blood in the syringe was put on ice in a cooler box and taken within one hour after sampling to Ampath Trust (Pathology Laboratory services, Lyttelton, Centurion, South Africa) for blood gas analyses.

4.2.2.2. Volatile fatty acid determination

Four mL of the filtered rumen digesta was preserved with 1 mL of 25% H₃PO₄ (orthophosphoric acid) and then stored at -10 °C for VFA analysis using gas chromatography (Gibbs *et al.*, 1973). Thawed ruminal fluid samples were clarified by centrifugation at 18,457 × *g* at 4°C for 20 min before analyzing. Volatile fatty acids were identified and quantified from chromatograph peak areas using calibration with external standards.

4.2.2.3. Determination of blood gases

Bicarbonates (HCO₃), and base excess (BE) were determined with a pH/blood gas analyzer (BAYER Rapid Lab 865; Siemens Healthcare Diagnostics Inc., Deerfield, IL) as described by Brossard *et al.* (2003). Blood partial pressure of the gaseous O₂ dissolved in blood (PO₂), and partial pressure of CO₂ (PCO₂), were measured by a semi-automatic system (Ciba-Corning 278 Blood Gas System, Medfield, MA) adjusted for hematocrit.

4.2.2.4. Bacteria count

The population size of *M. elsdenii* NCIMB 41125 was determined to confirm its colonization and establishment in the digestive tract. Total bacteria and *M. elsdenii* count were also determined to evaluate the relationship with *M. elsdenii* NCIMB 41125. The DNA from rumen fluid samples and from bacterial cultures was extracted by physical disruption using a bead beater (Mini-Beater; BioSpec Products, Bartlesville, OK, USA) following the protocol described by Whitford *et al.* (1998). The 16S rRNA gene was enzymatically amplified from genomic DNA using PCR methods previously described by Ouwerkerk and Klieve (2001). Sequencing was performed using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq® DNA Polymerase FS and a model 373 A DNA sequencing system (PE Applied Biosystems Inc., Foster City, CA, USA). Sequence fragments were assembled using Sequence Navigator (PE Applied Biosystems Inc). The Gapped Basic Local Alignment Search Tool (BLAST) database search programme (Altschul *et al.* 1990) was used to compare sequences.

4.2.3. Statistical analysis

Bacteria, *M. elsdenii* and *M. elsdenii* NCIMB 41125 counts were performed in order to confirm colonization. No statistical analysis was performed.

Mixed-effects linear regressions adjusted for treatment effects of each individual calf (n=24) and time of sampling (n=48) were performed to establish relationships between: 1) Total bacteria, *M. elsdenii* and *M. elsdenii* NCIMB 41125 population and 2) Ruminal volatile fatty acid, ruminal *M. elsdenii* and *M. elsdenii* NCIMB 41125 population using SAS (2009).

Means of ruminal molar percentage of VFA's (acetate, propionate and butyrate), acetate to propionate (A:P), acetate to butyrate (A:B) and acetate to propionate + butyrate (A:P+B) ratio of calves sampled (2 calves x 2 samples : n=4) at +24 and +72 hours relative to dosing within age group (7, 14 or 21 d) were statistically compared using independent two t-test, recommended for small sample size (de Winter, 2013). Statistical analyses were performed using SAS (2009). The model was:

$$t = \frac{(\bar{x}_1 - \bar{x}_2) - m}{\sqrt{s^2 \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}}$$

Where:

m = difference between means,

\bar{x}_1 = sample mean for the first sample,

\bar{x}_2 = sample mean for the second sample,

n_1 = sample size for the first sample, and

n_2 = sample size for the second sample.

The pooled sample standard deviation s is given by

$$s^2 = \left[\frac{(n_1 - 1) s_1^2 + (n_2 - 1) s_2^2}{(n_1 + n_2 - 2)} \right]$$

Significance was reported at $P < 0.05$ and tendencies at $P < 0.10$.

4.3. Results and discussion

4.3.1. Cell count of total bacteria, *M. elsdenii* and *M. elsdenii* NCIMB 41125

Fermentation end products such as volatile fatty acids and microbial protein are the result of the degradation of feed ingredients by the microbial population in the rumen (Castillo-González et al., 2014). The function of ruminal bacteria is associated with their much larger biomass and higher activity (Koike et al., 2009). Their specific role and function is determined by their presence in number in the digestive tract, which indicate their survival to the environment (Bryant MP, 1959). Thus, the availability of nutrients for absorption from the digestive tract depend on population sizes and species of bacteria in the rumen, with direct impact on animal performance. *Megasphaera elsdenii* NCIMB 41125, as well as *M. elsdenii* and total bacteria counts were determined and results are presented in Tables 4.4, 4.5, and 4.6. Bacteria counts were simply performed to confirm colonization by *M. elsdenii* NCIMB 41125 after an oral dose.

Table 4.4. Average bacteria counts (cfu/ml) in rumen digesta samples of pre-weaned Holstein calves not dosed or dosed with *M. elsdenii* NCIMB 41125 on day 7, 14 or 21 of age.

	Time relative to dosing Me	Treatments					
		7 days old calves		14 days old calves		21 days old calves	
		Not dosed	Dosed	Not dosed	Dosed	Not dosed	Dosed
Total bacteria	+24H	1.8 x 10 ¹¹	2.0 x 10 ¹¹	5.4 x 10 ¹¹	4.1 x 10 ¹¹	1.2 x 10 ¹²	7.1 x 10 ¹¹
	+72H	2.0 x 10 ¹¹	2.6 x 10 ¹¹	6.8 x 10 ¹¹	8.0 x 10 ¹¹	5.7 x 10 ¹¹	4.5 x 10 ¹¹
<i>M. elsdenii</i>	+24H	1.7 x 10 ⁶	3.3 x 10 ⁹	7.5 x 10 ⁹	1.2 x 10 ¹⁰	1.9 x 10 ¹⁰	1.1 x 10 ¹⁰
	+72H	5.2 x 10 ⁹	1.8 x 10 ¹⁰	2.9 x 10 ⁹	5.7 x 10 ¹⁰	1.3 x 10 ¹⁰	4.8 x 10 ⁹
<i>M. elsdenii</i> NCIMB 41125	+24H	ND ¹	7.3 x 10 ⁸	2.5 x 10 ⁶	3.2 x 10 ⁷	1.2 x 10 ⁸	4.3 x 10 ⁹
	+72H	2.2 x 10 ⁶	4.9 x 10 ⁹	1.6 x 10 ⁶	1.4 x 10 ⁹	1.9 x 10 ⁷	7.6 x 10 ⁷

¹: None detectable

Development of the rumen microorganisms determine fermentation end products as well as the structural and physiological properties of the rumen (Klein et al., 1987; Beharka et al., 1998). In the present study, total bacteria counts in the rumen were within the range (3.3 x 10⁹ CFU/ml; 10⁹-10¹² CFU/ml; 10¹⁰-10¹¹ CFU /ml) reported by Bryant et al. (1958), Fuller (1992) and Hespell et al., (1997), respectively. Bryant et al. (1958) reported earlier that the lactate utilising bacteria count in the rumen of a 3 week old calf averaged 3.1 x 10⁹ CFU/ml. In the present

study, the count of *M. elsdenii*, the more predominant lactate utilizing bacteria ranged between 10^9 and 10^{10} CFU/ml) at both +24 h and +72 h in the rumen of 7, 14 and 21 days old dosed calves. In the present study, *M. elsdenii* NCIMB 41125 counts in the faeces, colon and rumen were in the order of 10^6 cfu/ml as reported by McDaniel et al. (2009) in steers after an oral dose.

Ruminal total bacteria numbers at +24 h and +72 h in all calves (dosed and not dosed) ranged from 2.4×10^{11} to 1.1×10^{12} cfu/ml. *Megasphaera elsdenii* numbers ranged from 4.1×10^7 to 9.9×10^8 cfu/ml. As in faeces, *M. elsdenii* NCIMB 41125 was detected in the colon digesta sample of 7 and 14 days dosed calves at both +24 h and +72 h, but not in control calves. However in 21 days old calves it was detected in both dosed and control calves. *Megasphaera elsdenii* NCIMB 41125 count in the colon of all calves ranged from 1.4×10^6 to 1.3×10^8 cfu/ml.

Table 4.5. Average bacteria counts (cfu/ml) in colon digesta samples of pre-weaned Holstein calves not dosed or dosed with *M. elsdenii* NCIMB 41125 on day 7, 14 or 21 of age.

	Time relative to dosing Me	Treatments					
		7 days old calves		14 days old calves		21 days old calves	
		Not dosed	Dosed	Not dosed	Dosed	Not dosed	Dosed
Total bacteria	+24H		3.0×10^{11}	1.4×10^{11}	3.6×10^{11}	1.1×10^{12}	1.1×10^{12}
	+72H	1.3×10^{10}	2.4×10^{11}	8.3×10^{11}	1.1×10^{12}	1.7×10^{12}	6.0×10^{11}
<i>M. elsdenii</i>	+24H		4.6×10^8	4.1×10^7	5.1×10^8	7.7×10^8	1.7×10^8
	+72H	1.8×10^9	5.8×10^8	1.8×10^8	9.9×10^8	2.6×10^8	4.8×10^7
<i>M. elsdenii</i> NCIMB 41125	+24H	ND ¹	1.1×10^8	ND	8.2×10^6	2.8×10^6	4.6×10^7
	+72H	ND	1.3×10^8	ND	1.9×10^7	1.1×10^7	1.4×10^6

1: None detected

Ruminal total bacteria numbers at +24 h and +72 h hours in dosed and not dosed calves (7, 14 and 21 days old) ranged from 1.8×10^{11} to 1.2×10^{12} cfu/ml. *Megasphaera elsdenii* numbers ranged from 1.7×10^6 to 4.8×10^{10} cfu/ml. As in faeces, *M. elsdenii* NCIMB 41125 was detected in rumen of dosed calves at both +24 h and +72 h, but also in control calves, and ranged from 1.6×10^6 to 4.9×10^9 cfu/ml.

Table 4.6. Average bacteria counts (cfu/ml) in faecal samples of pre-weaned Holstein calves not dosed or dosed with *M. elsdenii* NCIMB 41125 on day 14 of age.

Count (cfu/ml)	Time relative to dosing Me (hour)	Treatments	
		Not dosed (n=4)	Dosed (n=4)
Total Bacterial	-24H	1.6 x 10 ¹¹	8.4 x 10 ¹¹
	+24H	4.2 x 10 ¹¹	7.9 x 10 ¹¹
	+72H	7.3 x 10 ¹¹	9.5 x 10 ¹¹
<i>M. elsdenii</i>	-24H	1.2 x 10 ⁹	3.0 x 10 ⁹
	+24H	1.3 x 10 ⁸	4.0 x 10 ⁹
	+72H	1.5 x 10 ⁹	1.5 x 10 ⁹
<i>M. elsdenii</i> NCIMB 41125	-24H	ND ¹	3.5 x 10 ⁶
	+24H	ND	3.5 x 10 ⁸
	+72H	ND	1.2 x 10 ⁷

¹: Not detectable

Faecal total bacteria numbers at -24 h as well as +24 h and +72H hours in both 14 days old dosed and not dosed calves ranged from 1.6 x 10¹¹ to 9.5 x 10¹¹ CFU/ml. *Megasphaera elsdenii* numbers ranged from 1.3 x 10⁸ to 4.0 x 10⁹. The total bacteria count and *M. elsdenii* count in dosed calves were numerically higher than those found in control calves. *Megasphaera elsdenii* NCIMB 41125 was detected in faecal samples of dosed calves at both +24 h and +72 h, but not in control calves. *Megasphaera elsdenii* number was lower at +24h and *M. elsdenii* NCIMB 41125 number lower at +24h and +72h in treated calves than those of control calves, but the opposite was observed for total bacteria at the same time. One could speculate that the population of the strain tested for might decline with time. In one calf, *M. elsdenii* NCIMB 41125 was detected before actually being dosed with *M. elsdenii* NCIMB 41125 (-24 h), probably due to cross contamination.

The purpose of direct fed microbial supplements is to facilitate the establishment and maintenance of a suitable microbial flora population in the gastrointestinal tract (Agarwal et al., 2002). When DFM's are given orally, their colonization in the rumen is a prerequisite for their function and their expression depends on their tolerance towards gut environment (Agarwal et al., 2002) and is an important criteria for establishment in the rumen. *Megasphaera elsdenii* NCIMB 41125 was earlier characterized as having a high growth rate (up to 0.938/h) (Horn et al, 2009), with a biomass accumulation that provides more tolerance to environment stressors (Meissner et al, 2010), with minimal activity and replication ability at a pH range of 4.5-5.5 (Horn et al, 2009). Detection of the strain NCIMB 41125 is an indication of its potential to colonize in the digestive tract of calves. The detection of the strain in some control calves

can be attributed to cross contamination, although strict precautions were taken to prevent cross contamination.

The mixed-effects linear regressions to determine whether the change in total bacteria and/or *M. elsdenii* were due to the *M. elsdenii* NCIMB 41125 population indicated no relationship between *M. elsdenii* NCIMB 41125 and total bacteria count ($P= 0.54$) (Data not shown). Instead, the mixed-effects linear regressions indicated that there was a strong positive relationship ($R^2=0.96$; $P < 0.0001$) between *M. elsdenii* NCIMB 41125 and *M. elsdenii* population (cfu/ml) in the rumen, suggesting that the increase in *M. elsdenii* was due to increased *M. elsdenii* NCIMB 41125, and the equation was:

$M. Elsdenii$ (cfu/ml) = $4.40E+09 + 41.1811 M. elsdenii$ NCIMB 41125 (cfu/ml) (Figure 4.2).

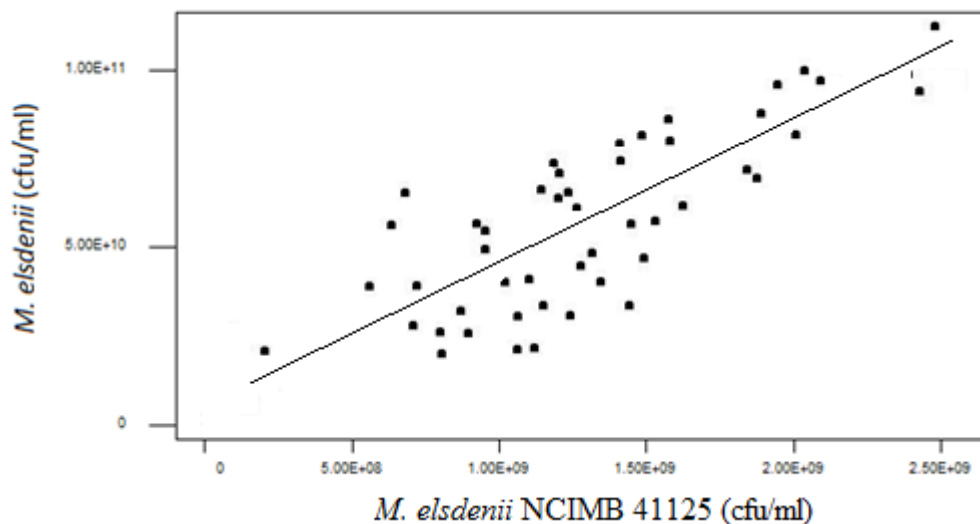


Figure 4.2. Relationship for random effect of each calf and time of sampling, between ruminal *M. elsdenii* NCIMB 41125 (Me) and total ruminal *M. elsdenii*, (cfu/ml)

These results suggest that *M. elsdenii* NCIMB 41125 colonize both the rumen and colon thereby increasing the cell counts of *M. elsdenii* and *M. elsdenii* NCIMB 41125 with little or no change in the total bacteria count. No colon digesta samples from treatment Me0 7 day +24 h were collected due to empty colons.

4.3.2. Effects of *M. elsdenii* NCIMB 41125 on rumen volatile fatty acid

Results on molar percentage of the primary VFA's (acetate, propionate and butyrate) are presented in Table 4.7. There was no difference ($P>0.05$) in molar percentage of acetate

between 7 day old calves dosed and not dosed with *M. elsdenii* NCIMB 41125 at both +24 and +72 h after oral dosing. Propionate was lower ($P < 0.001$) in dosed calves compared to control calves at +24 h, but no difference was observed at +72 h, although it was still numerically lower in dosed calves. The opposite was true for butyrate as it did not differ between the two groups of calves at +24 h, but was almost 150% higher ($P < 0.001$) in dosed calves compared to control calves at +72 h.

Kristensen et al. (2007) evaluated VFA's proportion in rumen of calves from 7 to 56 days and reported average molar percentage of acetate, propionate and butyrate of 52, 34 and 9 in 7 days old calves and 56, 32 and 8 for 21 day old calves, respectively.

Table 4.7. Molar percentage of acetate, propionate and butyrate in calf rumens of different ages at 24 and 72 h.

VFA	Calf age	Time	Control calves	Dosed calves	SEM	P
Acetate molar %	7 days	+24H	51.8	45.9	1.71	0.17
		+72H	36.4	39.5	1.32	0.15
	14 days	+24H	39.6	47.4	1.35	< 0.001
		+72H	61.4	33.2	1.73	< 0.001
	21 days	+24H	54.9	59.9	1.81	0.28
		+72H	52.8	55.6	9.68	0.19
Propionate molar %	7 days	+24H	18.2	9.5	0.81	< 0.001
		+72H	18.9	12.2	0.93	0.61
	14 days	+24H	27.2	25.9	0.59	0.19
		+72H	21.9	18.7	0.83	0.15
	21 days	+24H	34.0	27.9	1.17	< 0.001
		+72H	25.7	22.6	3.23	0.17
Butyrate molar %	7 days	+24H	12.1	8.9	0.62	0.83
		+72H	10.0	24.3	0.63	< 0.001
	14 days	+24H	8.5	9.1	1.12	0.24
		+72H	6.8	20.4	0.79	< 0.001
	21 days	+24H	8.3	6.7	2.90	0.12
		+72H	9.2	10.9	2.15	0.15

Values in the same row differ if $P < 0.05$ and tend to differ if $P < 0.10$.

In the present study, molar percentage of acetate and propionate in 7 d control calves were similar to values reported by Kristensen et al (2007) for 14 d calves, while for dosed calves values were lower, although difference was observed only for propionate at +24 h. Butyrate in both groups was close to the values observed by Kristensen et al. (2007) but the effect of dosing with *M. elsdenii* NCIMB 41125 resulted in more than double increase in proportion at +72 h.

In 14 days old calves, acetate was higher in dosed calves 24 h after dosing, but was decreased at +72 h compared to control calves of the same age. Molar % of propionate in dosed calves did not differ ($P>0.05$) from calves not dosed at both +24 and +72 h after dosing. As for 7 days old calves, butyrate was similar in 14 days old dosed and control calves at +24 h, but higher ($P<0.05$) in dosed calves compared to control calves at +72 h. In these 14 d old calves acetate proportion at +24 h for both groups was close to values reported by Kristensen et al. (2007) for calves of the same age, and as for 7 d old calves, dosing *M. elsdenii* NCIMB 41125 increased the proportion of butyrate at +72 h ($P < 0.05$).

In 21 day old calves, propionate was higher ($P<0.001$) when measured at +24 h in control calves compared to dosed calves, but did not differ between the two groups at +72 h. Butyrate was similar ($P>0.05$) in both groups of 21 days old calves at +24 and +72 h. Molar percentage of acetate, propionate and butyrate were also similar to values reported by Kristensen et al. (2007), and were not affected by orally dosing *M. elsdenii* NCIMB 41125, except for propionate at +24 h ($P < 0.05$).

Propionate was lower ($P<0.001$) in dosed calves both at 24 and 72 h after every dosing in 7, and 21 days old calves, but did not change ($P>0.05$) in 14 day old calves compared to control calves. An increase in molar % of butyrate ($>100\%$ improvement) was observed from +24 h to +72 h when calves were dosed on both d 7 and 14, while an increase occurred when calves were dosed on day 21. Acetate declined less from +24 h to +72 h in 7 days, but more in 21 day old calves.

Ratios of VFA's in control and dosed calves for 7, 14 and 21 day old calves were calculated and presented in Table 4.8. Changes in ruminal VFA concentrations varied across groups and time of sampling. The A:P was found to be higher in 7 d old dosed calves at +24 h and +72 h, and higher only at + 72 h in 21 d old calves compared to control calves. When 7 d old calves were dosed, A:P was lower at +72 h compared to control calves. The A:B ratio was found to be lower at +72 h in both 7 and 14 d old calves, while the P:B was lower at +72 h in all ages (7, 14 and 21 d) of dosed calves, The ratio A:(P+B) increased with dosing at 7 and 14 d ($P<0.05$) and 21 d (numerically) when measured after 24 h. In addition, A:(P+B) decreased ($P<0.001$) when calves were dosed at 14 d, but only numerically when dosed at 7 and 21 d. These results suggest that under these conditions, *M. elsdenii* altered the rumen fermentation patterns in favour of butyrate, suggesting potential benefits for rumen development.

Studies on effects of *M. elsdenii* NCIMB 41125 in pre-weaned dairy calves are lacking. The shift towards butyric acid has been observed in studies with post-weaned animals and was attributed to decreased rumen pH. Counotte et al. (1981) and Marounaek et al., (1989) reported that when pH decreased, fermentation shifted from propionate acid to butyric acid and valeric acid.

Table 4.8. Volatile fatty acids ratio in the rumen of calves dosed with *M. elsdenii* NCIMB 41125.

	Calf	Time	Control	Dosed	SEM	P
A:P	7 days	+24H	2.9	4.8	0.08	<0.01
		+72H	1.9	3.3	0.07	<0.01
	14	+24H	1.4	1.8	0.03	<0.01
		+72H	2.8	1.8	0.05	<0.01
	21	+24H	1.6	2.0	0.01	<0.01
		+72H	2.1	2.5	0.04	0.10
A:B	7 days	+24H	2.1	5.1	0.21	<0.01
		+72H	3.6	1.6	0.15	<0.01
	14	+24H	4.0	5.2	0.22	0.13
		+72H	9.3	1.6	0.70	<0.01
	21	+24H	7.0	9.1	1.50	0.39
		+72H	5.7	5.2	0.80	0.67
P:B	7 days	+24H	0.7	1.1	0.21	<0.01
		+72H	1.9	0.5	0.15	<0.01
	14	+24H	3.2	2.9	0.20	0.07
		+72H	3.2	0.9	0.55	0.02
	21	+24H	4.1	4.1	0.81	0.39
		+72H	2.5	2.0	0.24	0.04
A:(P+B)	7 days	+24H	1.7	2.5	1.01	0.001
		+72H	1.3	1.1	0.85	0.246
	14	+24H	1.1	1.4	0.80	0.020
		+72H	2.1	0.8	1.07	< 0.001
	21	+24H	1.3	1.7	0.44	2.130
		+72H	1.5	1.7	1.83	0.594

A:P: Acetate to propionate ratio; A:B Acetate to butyrate ratio; P:B: Propionate to butyrate ratio. Values in the same row differ if $P < 0.05$ and tend to differ if $P < 0.10$.

Henning et al. (2010a) also reported a shift towards butyric and valeric acid in vivo (feedlot diets) at an even lower pH range. With the same strain, Hagg et al. (2010) found no significant difference between dairy cows dosed with *M. elsdenii* NCIMB 41125 and control cows when fed TMR's with an average rumen pH of around 5.8. With rumen pH remaining around 6, *M. elsdenii* NCIMB 41125 shifted fermentation to more propionic acid and lower A:P in dairy cows (Aikman, 2008; Aikman et al., 2009). In a continuous culture study of the ruminal

bacterium *Megasphaera elsdenii* using lactate as carbon source, butyric acid, the minor product in batch culture, was the main product (Cruz et al., 2001).

Mixed-effects linear regressions were performed in an attempt to determine whether an increased population of *M. elsdenii* NCIMB 41125 and/or *M. elsdenii* was the result of dosing with *M. elsdenii* NCIMB 41125 and whether dosing would contribute to rumen development of the population through increased ruminal molar % of butyrate and /or propionate,

There were no relationships between ruminal concentration of *M. elsdenii* NCIMB 41125 and molar percentage of VFA's, and between ruminal *M. elsdenii* and propionic acid, but ruminal *M. elsdenii* (cfu/ml) presented a positive relationship with ruminal molar percentage of butyrate ($P < 0.001$, $R^2 = 0.43$) and a concomitant negative relationship with ruminal acetate ($P = 0.017$, $R^2 = -0.33$). The equations were:

- Ruminal butyric acid (%) = $4.121 + 5.76E-11$ ruminal *M. elsdenii* (Figure 4.3)
- Ruminal acetic acid (%) = $26.454 - 9.30E-11$ ruminal *M. elsdenii* (Figure 4.4)

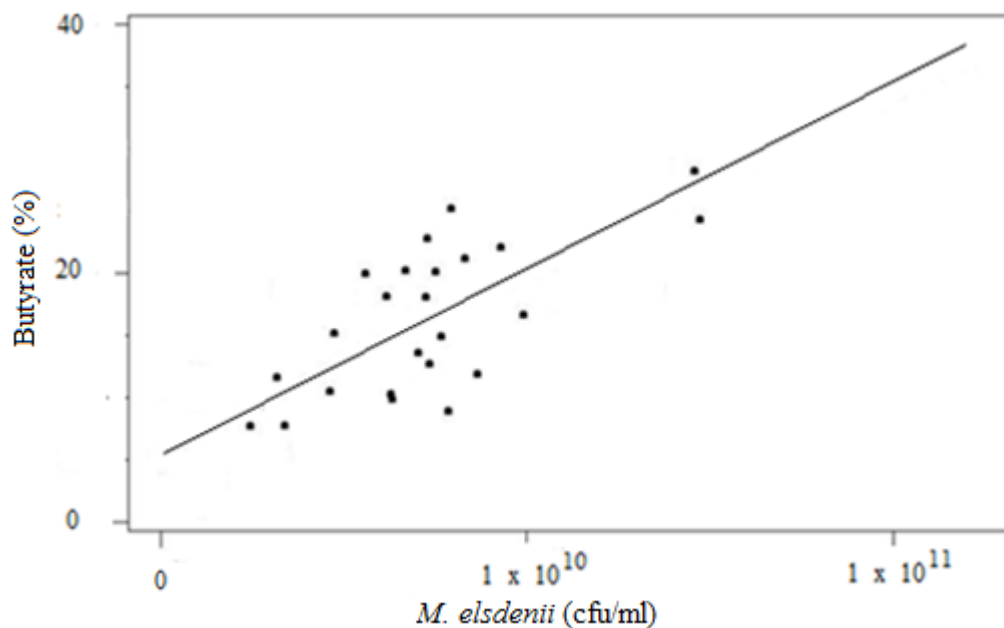


Figure 4.3. Relationship for random effect of each calf and time of sampling, between ruminal *M. elsdenii*, (cfu/ml) and ruminal molar percentage of butyrate (%).

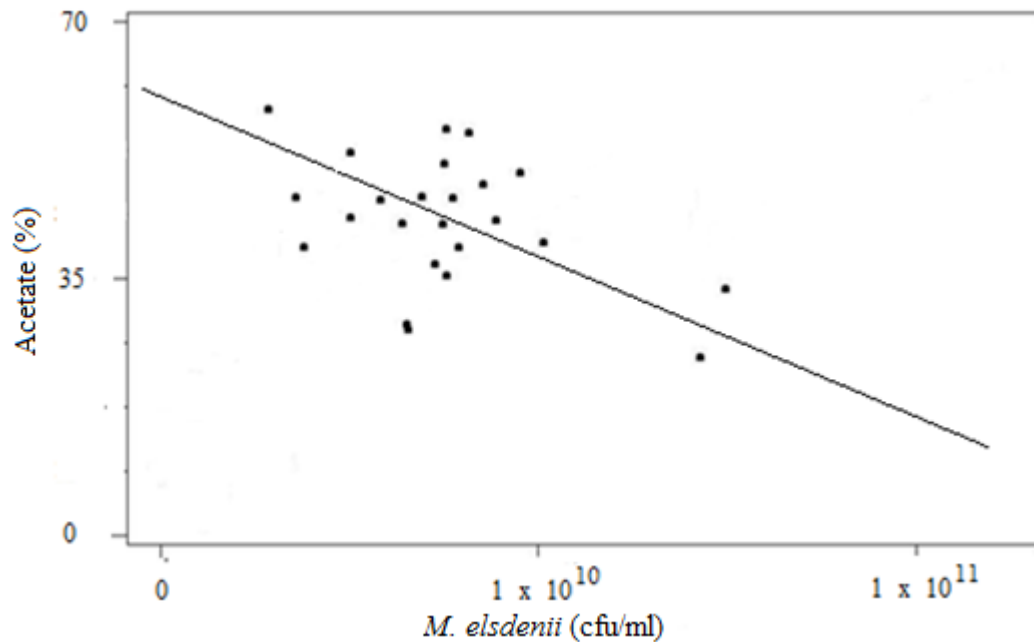


Figure 4.4. Relationship for random effect of each calf and time of sampling, between ruminal *M. elsdenii*, (cfu/ml) and ruminal molar percentage of acetate (%).

These results suggest that dosing pre-weaned dairy calves with *M. elsdenii* NCIMB 41125 has the potential to alter ruminal VFA production through increasing proportions of butyrate at the expense of propionate. Further research work with larger number of calves is warranted to confirm the effect of *M. elsdenii* NCIMB 41125 on rumen fermentation patterns and the potential impact on the total bacterial population.

4.3.3. Effects of *M. elsdenii* NCIMB 41125 on blood gases

The effect of *M. elsdenii* NCIMB 41125 dosing on blood metabolites before and after dosing is shown in Table 4.9 and Table 4.10 respectively.

Neonatal diarrhoea is a major cause of illness and death in calves less than 1 month of age. Blood gases are indicators of the state of metabolic acidosis and dehydration caused by calf diarrhoea and their measurement is useful for assessing acid–base disorders (Gomez et al., 2013). Blood gases were determined to see whether dosing with *M. elsdenii* NCIMB 41125 would affect the metabolic status of calves with naturally occurring diarrhea. Mean blood pH values were in the range reported as normal for calves by Kasari and Naylor (1984) and Nagy et al. (2003) (7.36 -7.43), but in those studies only a few calves had pH > 7.43. Higher values

approaching alkalaemia (pH 7.45 and more), were also reported by Vestweber et al. (1977) and Verhoeff et al. (1985).

Table 4.9. Blood metabolites in calves 2 hours before dosing with *M. elsdenii* NCIMB 41125

Parameters	Treatments					
	7 days old calves		14 days old calves		21 days old calves	
	Me0	Me7	Me0	Me14	Me0	Me21
pH	7.28	7.30	7.36	7.42	7.29	7.37
Lactate	2.58	2.28	1.70	1.30	2.55	4.15
PCO ₂ , mmHg	41.25	46.5	47.25	39.75	42.50	42.00
HCO ₃ , mmol/l	25.85	29.25	25.35	25.50	27.17	23.83
PO ₂ , mmHg	56.00	56.50	54.00	65.50	77.50	68.00
BE, mmol/l	1.23	4.08	-0.28	0.93	1.70	4.53

PCO₂: partial pressure of CO₂; HCO₃: Bicarbonates; PO₂: partial pressure of O₂; BE: base excess

Reece and Hotchkiss (1987) suggested that because of diverse concentrations of basic components in milk replacer or whole milk, metabolic alkalosis may occur, and is associated with production and secretion of H⁺ ions into the abomasal content, which is accompanied by production and absorption of bicarbonate ions into blood. Regardless of the causal agents, diarrhea in neonates is often complicated by metabolic acidosis (Grove-White and White, 1993). According to the range (46-64 mmHg) suggested by Butler et al. (1971), except for background and PCO₂ at 2 h before dosing, blood PCO₂ was low in all the samples. Calves dosed with *M. elsdenii* NCIMB 41125 had normal blood HCO₃ at all the sampling times. Seven day old control calves sampled at +24 h and 21 days old control calves sampled at +24 h had normal blood HCO₃ and the rest was out of the normal range. As for PCO₂, most of blood HCO₃ of calves was below the normal range (24-34 mmol/l) suggested by Butler et al. (1971). This situation suggests that the majority of calves were having diarrhoea and that large amounts of water and electrolytes were lost from the body causing a change in acid-base status resulting in an electrolyte imbalance. To compensate for loss of HCO₃ during diarrhoea, high levels of CO₂ is excreted through the lungs (hyperventilation), which causes a decreased PCO₂ (Costello, 2007). This is confirmed by the positive correlation observed between PCO₂ and HCO₃ (Table 4.11). Blood bicarbonate concentrations also depends on the amount of secreted H⁺ ions needed for reduction of abomasal pH to normal values (Reece and Hotchkiss, 1987). Newborn calves experience tremendous stress after removal from the dam and exposure to a new environment (Besser and Gay, 1994). One of the major mechanisms by which the intestinal

tract of a newborn calf reacts to pathogenic bacteria or viruses or indigestible dietary nutrients is hyper secretion and a relative lack of intestinal absorption which results in a loss of fluids, electrolytes, and nutrients, and the net effect is diarrhea (Radostits, 1974).

Overall, no blood parameter value has shown any major difference between treatments indicating that dosing *M. elsdenii* NCIMB 41125 did not affect these values. Only L-lactate was determined in the present study, preferably, both L and D-lactate must be determined for better interpretation of results.

Table 4.10. Mean blood metabolites in calves after dosing with *M. elsdenii* NCIMB 41125

Parameters	Treatments					
	7 day old calves		14 day old calves		21 day old calves	
	Me0	Me7	Me0	Me14	Me0	Me21
pH	7.40	7.43	7.40	7.41	7.46	7.42
Lactate	2.01	3.49	1.44	2.25	2.72	1.67
PCO ₂ , mmHg	43.25	44.25	39.12	38.75	35.66	38.25
HCO ₃ , mmol/l	58.65	60.0	69.15	70.5	75.65	69.90
PO ₂ , mmHg	26.36	28.71	23.87	24.39	25.26	24.24
BE, mmol/l	1.34	3.81	-0.76	-0.12	1.78	-0.14

PCO₂: partial pressure of CO₂; HCO₃: Bicarbonates; PO₂: partial pressure of O₂; BE: base excess

The correlation matrix between blood metabolites of calves dosed or not with *M. elsdenii* NCIMB 41125 are presented in Table 4.11.

Table 4.11. Correlation matrix between blood metabolites from all calves (n=24)

Variables	pH	Lactate	PCO ₂	PO ₂	HCO ₃	Base excess
pH	1	0.053	-0.140	0.143	-0.103	0.087
Lactate	0.053	1	-0.067	-0.011	-0.077	-0.039
PCO ₂	-0.140	-0.067	1	-0.545	0.764	0.484
PO ₂	0.143	-0.011	-0.545	1	-0.411	-0.208
HCO ₃	-0.103	-0.077	0.764	-0.411	1	0.761
Base excess	0.087	-0.039	0.484	-0.208	0.761	1

Values in bold and *italic* are significantly different ($P < 0.05$) and tend to differ ($P < 0.10$), respectively, from 0.

PCO₂: partial pressure of CO₂; HCO₃: Bicarbonates; PO₂: partial pressure of O₂; BE: base excess

Lactate was negatively correlated with HCO₃ (Table 4.11). Metabolic acidosis in diarrheic calves seem to develop from the combination of large fecal bicarbonate ion losses (Tennant et al., 1972; Lewis and Phillips, 1972) and plasma lactic acid accumulation (Tennant et al., 1972; Lewis et al., 1975) and can be associated with a loss of bicarbonate. Increased lactate indicates an insufficient supply of oxygen to the body and tissue hypoxia with consequently increased anaerobic glycolysis. Lactate was positively correlated with the pH, which does not agree with Thielscher (1994) and Steinhardt et al. (1995) who indicated that increased lactate was associated with increased concentrations of H⁺ ions and decreased blood pH.

4.4. Conclusion

The results of this pilot study suggest that *M. elsdenii* NCIMB 41125 colonize both the rumen and colon of pre-weaned calves following an oral dose, increasing the cell count of *M. elsdenii* and *M. elsdenii* NCIMB 41125 with little or no change in total bacteria count. Dosing *M. elsdenii* NCIMB 41125 on d 7 and d 14 altered the rumen fermentation patterns resulting in high plasma butyrate measured 72 hours later, suggesting potential benefits for rumen development. In general, blood metabolites of all calves were within the accepted ranges and dosing with *M. elsdenii* NCIMB 41125 did not show any noticeable effects on blood gases. Further research is necessary to better understand the relationship between dosing calves with *M. elsdenii* NCIMB 41125 and its effects on digestive tract colonization, VFA concentrations and blood metabolites

CHAPTER 5

EFFECTS OF DOSING *MEGASPHAERA ELSDENII* NCIMB 41125 ON FEED INTAKE, GROWTH AND SOME HEALTH PARAMETERS IN PRE-WEANED HOLSTEIN CALVES IN A CONVENTIONAL CALF REARING SYSTEM

5.1. Introduction

Early weaning of newborn dairy calves necessitates changes in production and management systems toward accelerated ruminal development (Guilloteau et al., 2009). Rumen epithelial and papillae development in pre-weaned ruminants is stimulated by butyrate and propionate (Coverdale et al., 2004) and the speedy initiation of solid food intake, is vital to the calf. An early onset of rumen development requires sufficient solid feed intake for successfully making the transition from a pre-ruminant to a functioning ruminant (Coverdale et al., 2004). This could allow producers to wean calves that have a more mature and developed digestive systems. *Megasphaera elsdenii* has potential to play a major role in production of branched-chain volatile fatty acid (VFAs) in the rumen (Wallace, 1986) and may ferment feed to propionic acid as end product, but sometimes butyric acid at the expense of propionic acid (Henning et al., 2010). The objectives of this study was to determine the effect of oral dose and time of dosing of *Megasphaera elsdenii* NCIMB 41125 on intake, structural growth and development, and occurrence of diarrhoea in pre-weaned dairy calves.

5.2. Materials and methods

5.2.1. Design and treatments

The experiment was conducted at the dairy research unit at the Agricultural Research Council, in Pretoria, South Africa and was approved by the Animal ethics committee of the Agricultural Research Council / Animal Production Institute (APIEC 12/012). Eighty Holstein calves were blocked on the basis of order of birth and sex and randomly allocated to one of four treatments. The experimental treatments consisted of a control group (Me0), and three *M. elsdenii* groups, that received a 50-mL oral dose of *M. elsdenii* NCIMB 41125 (10^8 CFU/mL) at 7 d (Me7), 14 d (Me14), or 21 d (Me21) of age. Calves remained in the experiment until weaned. Calves were eligible for weaning at 42 days if they were able to consume 1 kg of calf starter (90 % DM) or once they consumed 1 kg of calf starter during the period after day 42. If a calf experienced severe diarrhoea, the amount of milk fed was decreased to 50% of the normal allocated milk

until diarrhoea subsided. Animals were closely monitored for clinical signs of diarrhoea or other metabolic problems.

Calves received colostrum for 3 consecutive days after birth before being fed whole milk and a commercial calf starter in pellet form (CP: 181 g/kg of DM; Fat: 35 g/kg of DM; NDF: 219 g/kg DM; ME: 14.3 MJ/kg; Ca: 8.1 g/kg DM; P: 5.1 g/kg DM) (Afgri Animal Feeds: 12 Bvls Bridge Boulevard, Highveld, centurion South Africa). Calf starter was available *ad lib* from d 3 to the end of the trial. Fresh water was also available *ad lib* from d 3 to the end of the trial. The milk feeding regime is shown in Table 5.1.

Table 5.1. Milk¹ feeding regime of calves dosed or not with *M. elsdenii* NCIMB 41125

		Morning	Afternoon
Day 4 to day 7	Milk	2 litres	2 litres
Day 8 to day 14	Milk	3 litres	3 litres
Day 15 to day 35	Milk	2 litres	2 litres
From Day 36 to weaning	Milk	2 litres	-

¹ Average milk composition: CP: 3.23 %; fat: 3.47 %; lactose: 4.69 %

5.2.2. Measurements and sampling

Calf starter was sampled and analyzed for DM by oven drying at 60°C for 48 h. Dried samples were ground and analysed for crude protein (CP) according to AOAC, (2000) procedure 968.06 and ether extract according to AOAC (2000) procedure 920.39. Calcium (Ca) and potassium were determined according to Giron, (1973) using a Perkin Elmer Atomic Spectrophotometer. Phosphorus (P) was assayed according to AOAC (2000) procedure 965.17. The NDF was determined according to Van Soest et al. (1991). Composite daily milk samples from bulk tank collected in the morning and evening were collected monthly to determine fat, crude protein, and lactose at Lacto Lab (Pty) (Irene) using a System 4000 Infrared Analyser (Foss Electric, Hillerod, Denmark). Growth parameters (BW, chest circumference and hip width, hip height, shoulder height) were measured at birth and every seven days thereafter to determine overall tissue and skeletal growth. For BW, an electronic scale (Figure 5.1 and 5.2) with accuracy to the nearest 200 g (<50 kg) or the nearest 500g (>50 kg) was used. Remaining growth measurements were done with a measuring tape to the nearest 5mm. Shoulder height was

defined as the distance measured around the peak of the shoulder blades; hip height was defined as the distance directly over the point of the hip with the calf standing on level ground; hip width was the widest point at the centre of the stifle; chest was defined as the distance around chest directly under the armpits. Daily intake of calf starter, milk and water were determined from amount fed minus orts, and recorded.

All occurrences and treatments of lung diseases and diarrhoea were noted individually. Lung infection was defined as coughing or sneezing for 2 d or more, or as heavy breathing together with additional signs such as nasal discharge. Diarrhoea was defined as soft or watery faeces lasting for 2 d or more, or as soft or watery faeces in combination with an impaired general condition and possible weight loss (Gulliksen et al., 2009a).

Faecal scoring for the determination of faecal fluidity and consistency was conducted daily in the morning (08h00) with the score ranging from 1 to 4; 1 indicating formed stools, 2 when soft or of moderate consistency, 3 when runny or mild diarrhoea and 4 when watery and profuse diarrhoea (Larson et al. (1977). Number of days of diarrhoea (duration of diarrhoea, diarrhoea occurrence (frequency) and severity of diarrhoea (Faecal score) were recorded.

5.2.3 Statistical analysis

Variables measured daily were reduced to weekly means prior to analysis. All dependent variable data were processed using the procedure PROC MIXED of SAS (2009) and least squares means are reported. The statistical model included calf as a random effect, and experimental group and its interaction with time as a fixed effect. Initial BW was included in the model as a covariate. The model was subjected to an autoregressive order one. The statistical model used was

$$Y_{cgt} = \mu + \alpha g + \beta t + (\alpha\beta)gt + \gamma(\alpha)cg + ecgt,$$

Where:

Y_{cgt} = an observation value for BW, starter DMI, milk and water intake, parameters of structural growth and health status, measured from calf c from group g at time t ;

μ = overall mean for the population;

αg = fixed effect of group g , where g = group Me0, Me7, Me14 or Me21;

βt = fixed effect of time t , where t = w 1, 2, 3, 4, 5 or 6;

$(\alpha\beta)gt$ = fixed interaction of effect of group g and time t ;

$\gamma(\alpha)cg$ = random effect of calf c nested within group g ; and

$ecgt$ = error associated with the measurement taken from calf c from group g at time t .

Body weight, hip height, and shoulder height response variables measured at birth were included into the model as a covariate. Values were reported as least square means. Weekly averages were analysed as repeated measures using a second-order quadratic polynomial fitted to treatment day test at 5% significance. Significance was reported at $P < 0.05$ and tendencies at $P < 0.10$.

Means for initial and weaning BW and ADG, days of diarrhea, diarrhea occurrence and occurrence of lung diseases were subjected to ANOVA using PROC GLM (SAS Institute, 2009). Least square means are reported. The statistical model used was

$$Y_{ci} = \mu + T_i + \delta_c + e_{ci}$$

Where Y_{ci} = observation value for initial and weaning BW and ADG, days of diarrhea, diarrhea occurrence, severity of diarrhea and occurrence lungs infection taken from calf c at t time.

μ = overall mean of the population,

T_i = fixed effect of the i^{th} treatment (Me0, Me7, Me14 or Me21),

δ_c = random effect of calf, and

e_{ci} = error associated with the measurement taken from calf c from i^{th} treatment

Significance was reported at $P < 0.05$ and tendencies at $P < 0.10$.

5.3. Results and discussion

5.3.1. Calf starter intake

Average calf starter DMI ranged from 0.20 for control calves to 0.28 kg/d for Me-14 calves and was higher ($P < 0.05$) in the Me7 and Me14 treatments compared to calves not dosed, but did not differ between Me21 and Me0 treatments (Table 5.2). When evaluated in 3 different sub-periods, from d 7 to d 42 starter DMI was higher ($P < 0.05$) in Me7 and Me14, but did not

change in Me21 when compared to Me0. From d 14 to d 42 and from d 21 to d 42, intake of starter was higher ($P < 0.05$) in Me7 and Me14 calves compared to calves not dosed or dosed at day 21.

Table 5.2. Least square means of effects of *M. elsdenii* NCIMB 41125 on calf starter and water intake and days to weaning of milk fed dairy calves.

Items	Treatments ¹				SEM
	Me0	Me7	Me14	Me21	
Starter DMI (kg/d)	0.20 ^c	0.28 ^{ab}	0.28 ^a	0.22 ^{bc}	0.25
From d 7 to d 42 (kg)	215.7 ^b	296.6 ^{ac}	302.7 ^a	234.0 ^{bc}	27.45
From d 14 to d 42 (kg)	289.1 ^b	394.4 ^a	391.7 ^a	281.09 ^b	31.95
From d 21 to d 42 (kg)	360.0 ^b	483.0 ^a	483.9 ^a	363.2 ^b	39.46
² Total DMI, kg/d	0.73 ^c	0.81 ^{ab}	0.80 ^a	0.76 ^{bc}	0.36
Water Int (ℓ/d)	2.4 ^c	2.9 ^{ab}	3.0 ^a	2.5 ^{bc}	0.18
³ Days to Wean	43.8 ^{ab}	40.3 ^c	40.8 ^{bc}	45.4 ^a	1.60

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

¹Me0: No supplement; Me7: dosed at 7 days of age; Me14: dosed at 14 days of age; Me21: dosed at 21 days of age

²Total DMI: calf starter intake + milk intake

³Days to Wean: when consuming 1 kg starter (90 % DM)

Daily intake of water was also found to be higher ($P < 0.05$) in Me7 and Me14 compared to Me0 calves, and was associated with increased starter DMI in agreement with previous studies (Jenny et al., 1978; Kertz et al., 1984).

Days to weaning were less ($P < 0.05$) in Me7 compared to Me0 and Me21, less in Me14 compared to Me21, but did not differ between Me0, and Me14. Starter DMI (kg/d and kg/kg BW) was similar in all treatments in week 1 and 2 (Figure 5.1). A time effect ($P < 0.05$) was observed only from week 3 through the study. From week 3 to week 5, starter intake (kg/d) did not differ between Me7 and Me14 and between Me0 and Me21, remaining higher in the two first groups. During week 6, starter DMI (kg/d) was different between all treatments, being the highest in Me14, followed by Me7 and Me21 ($P < 0.05$).

The increasing effect of Me on DMI was observed a week after supplementation in both Me14 and Me21 calves, but only after two weeks in Me7 calves. The reason for the latter expression when dosed on day 7 may be due to the fact that the rumen at that stage was still underdeveloped compared to the rumen at 14 and 21 days of age. Dosing calves with *M. elsdenii* NCIMB 41125 on d 7 and 14 increased starter DMI by 38.1 and 41.0%, respectively compared to calves not

dosed. The higher intakes observed in calves receiving treatments Me7 and Me14 might be associated with an increase in the population of *M. elsdenii* NCIMB 41125 in the gut and an increase in ruminal butyric acid (Chap. 3).

Although no relevant literature could be found with *M. elsdenii* on dairy calves, previous studies have reported a positive effect of *M. elsdenii* on feed intake (Henning et al., 2009; Henning et al., 2010a) in lambs and steers drenched with *M. elsdenii* NCIMB 41125, respectively. Average feed intake of steers that received three different concentrations of *M. elsdenii* NCIMB 41125 showed an increase in feed intake of 21% (Henning et al., 2010a). Drouillard (2004) found that cattle drenched with strain 41125 tended to maintain higher intakes throughout the experiment compared to control cattle. In contrast no difference in feed intake was observed by Leeuw et al. (2009) in feedlot steers fed high and low roughage diets.

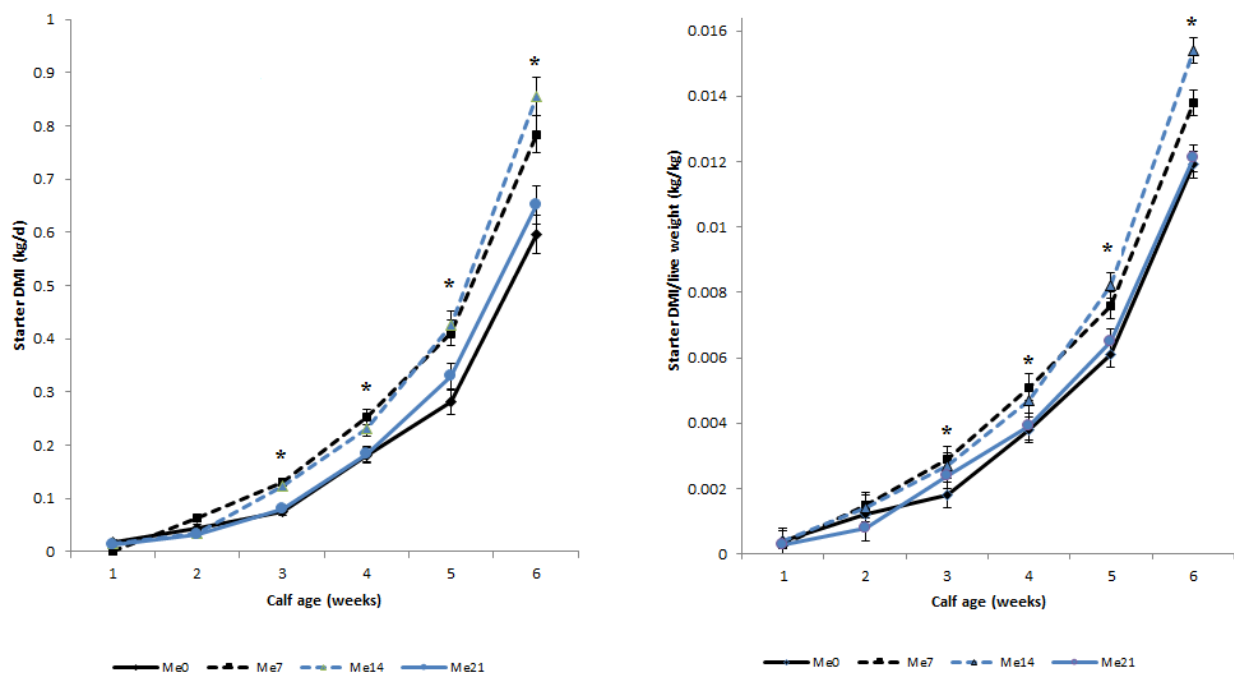


Figure 5.1. Starter DMI (\pm SE) (left) and starter DMI/kg BW (right) over period for Me0: No *M. elsdenii* (Me); Me7: Me supplemented at 7 days of age; Me14: Me supplemented at 14 days of age; Me2: Me supplemented 21 days of age. *Least square means differ significantly between treatment groups at the same time point ($P < 0.05$).

Dosing *Megasphaera elsdenii* increases propionate (Aikman, 2008; Aikman et al., 2009) and butyrate concentrations in the rumen (Marounek et al., 1989; Cruz et al., 2001) and it is generally accepted that butyrate and propionate are the primary VFA's responsible for rumen

epithelial and papillae development in the pre-weaned ruminant (Tamate et al., 1962; Coverdale et al., 2004). Butyric acid provides cell energy for the fueling processes for thickening of the rumen wall, formation of papillae, and increasing capillary development (Weigand et al., 1975), consequently increasing the capacity of solid feed intake (Coverdale et al., 2004). In a review of the effects of *M. elsdenii* NCIMB 41125, Meissner et al. (2010) concluded that feed intake is expected to be enhanced or to vary less when strain 41225 is administered. A possible explanation for the lack of difference in DMI between Me0 and Me21 calves is that *M. elsdenii* NCIMB 41125 was administered “too late” to affect an advantage above normal papillae development.

During week 3, DMI (kg/kg BW) was higher ($P < 0.05$) in Me14 compared to Me21 calves and tended ($P < 0.10$) to be higher in Me7 calves compared to Me0 and Me21. During week 4 and 5, DMI was higher in Me7 ($P = 0.10$) and Me14 ($P = 0.07$) compared to Me21, but all the treatments did not differ ($P > 0.05$) with the control calves (Me0). During the last week, DMI (kg/kg BW) was higher ($P < 0.05$) in Me14 followed by Me7 calves compared to the rest of treatments. No difference ($P > 0.05$) was observed between Me0 and Me21 calves.

Weaning is the transition from liquid to solid feed, and starter intake at weaning is essential to ensure rumen development and weaning calves successfully. Although calves in all groups increased starter DMI a week before weaning, it was more pronounced in Me7 and Me14-calves which could be attributed to a more developed rumen as speculated earlier. These results suggest that dosing calves with *Megasphaera elsdenii* NCIMB 41125 establish the organism in the GIT and benefit calves through increased DMI and earlier weaning. Calves dosed with *Megasphaera elsdenii* NCIMB 41125 on day 7 and 14 attained the required level of starter intake earlier improving weaning weight. The increase in starter DMI would be an advantage for weaned calves as it would help to avoid the weaning stress occurring in calves fed increasing amounts of milk (Quigley et al., 2006), and maintaining growth rates. This is supported by high intake (kg/d) and high starter intake per calf live weight during the last week prior weaning, which was associated with improved weaning BW.

5.3.2. Calf growth and efficiency

The effects of dosing *M. elsdenii* NCIMB 41125 on calf's growth, performance, skeletal development and health are presented in Tables 5.3 and 5.4 respectively.

Calves were not blocked on the basis of birth weight and resulted in initial body weight of the Me0 calves lower compared to the rest of the groups ($P < 0.05$). No differences in starting body weight were observed between Me7 and Me21 as well as between Me14 and Me21. Calves were considered eligible for weaning when consuming 1kg of calf starter per day. It was expected that the reduction of milk offered a week before weaning would have stimulated starter intake and increased weaning BW. At weaning, Me14 and Me7 calves were heavier than Me0 and Me21 calves.

Table 5.3. Least square means of effects of *M. elsdenii* on growth, performance and skeletal development of pre-weaned dairy calves

Items	Treatments ¹				SEM
	Me0	Me7	Me14	Me21	
Initial body Weight (kg)	36.0 ^b	39.6 ^a	40.7 ^a	39.5 ^a	1.19
Weaning BW (kg)	51.8 ^b	56.0 ^a	56.7 ^a	52.1 ^b	1.84
ADG (kg/d)	0.37	0.37	0.38	0.31	0.05
Gain:Feed (kg BW gain/kg DMI)	0.51 ^a	0.48 ^a	0.47 ^a	0.40 ^b	0.09
Shoulder height gain (cm)	6.3	7.1	7.0	6.3	0.58
Hip height gain (cm)	7.5	8.1	7.9	7.0	0.73
Hip Width gain (cm)	4.4	3.9	3.8	4.3	0.41
Chest gain (cm)	10.3 ^{bc}	9.4 ^{ab}	8.6 ^{ab}	8.0 ^a	0.63

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

¹Me0: No supplement; Me7: dosed at 7 days of age; Me14: dosed at 14 days of age; Me21: dosed at 21 days of age. ADG: Average daily weight gain.

However, ADG of calves was similar between groups. Efficiency of feed conversion (Gain:Feed) was lower for Me21 compared to the rest of treatments, and did not differ ($P > 0.05$) between Me0, Me7 and Me14, averaging 0.47, which is within the range of 0.55, 0.44 and 0.40 reported previously by Barlet (2001), Brown et al. (2005) and Khan et al. (2007), respectively for calves fed milk in a conventional rearing system. Shoulder height gain and hip height gain were not affected by treatments but were numerically higher in Me7 followed by Me14. No differences in hip width were observed between treatments. The chest circumference gain tended to be higher in Me0 ($P < 0.10$) when compared to Me21, but did not differ between Me0, Me7 and Me14 and between Me7, Me14 and Me21 treatments, respectively ($P < 0.05$).

Although dosing *M. elsdenii* NCIMB 41125 to 7 d and 14 d calves significantly increased starter intake, it did not translate into higher ADG, gain:feed or body structural measurements. This was somewhat unexpected and is difficult to explain.

5.3.3. Calf health

Mortality within treatments was recorded and 3, 2, 1 and 1 calf died from the groups receiving treatments Me0, Me7, Me14 and Me21 respectively. Diarrhoea is merely described as one of the common conditions affecting calves. During the experimental period, the presence of diarrhoea in the calves was determined by direct observation of the number of animals affected in each group. Over the 42-d trial period, the average numbers of days that diarrhoea was observed were 18 and 4.5% less in Me7 and Me14, and 11.4% higher in Me21 compared to Me0.

Table 5.4. Least square means of effects of no *M. elsdenii* (Me0), dosing *M. elsdenii* on day 7 (Me7), day 14 (Me14) and Day 21 (Me21) on health parameters of pre-weaned dairy calves

Items	Treatments ¹				SEM
	Me0	Me7	Me14	Me21	
Days of Diarrhoea	4.3	3.7	4.0	5.0	0.635
Diarrhoea occurrence	1.5	1.6	1.7	1.7	0.217
Occurrence of Lung diseases	0.2	0.3	0.2	0.05	0.105
Av body temperature (° C)	38.7	38.7	38.7	38.7	0.035
Faecal Score	2.2	2.2	2.2	2.3	0.045

¹Me0: No supplement; Me7: dosed at 7 days of age; Me14: dosed at 14 days of age; Me21: dosed at 21 days of age

Although the differences in days of diarrhoea were not significant, the observed decreased rates in Me7 and Me14 would have significant effects on associated antimicrobial treatment cost. No differences ($P > 0.10$) between treatments were observed for body temperature, faecal score and lung problems.

5.4. Conclusion

Dosing calves on day 7 and 14 after birth significantly increased calf starter dry matter intake and resulted in heavier calves at weaning at 42 days. The ADG and gain:feed ratio, however were not affected. Furthermore, dosing on d 7 decreased days to weaning. Results suggest that

supplementing calves with *Megasphaera elsdenii* NCIMB 41125 on day 7 or 14 after birth may benefit calves through increased starter intake and earlier weaning.

CHAPTER 6

EFFECTS OF DOSING *MEGASPHAERA ELSDENII* NCIMB 41125 ON PERFORMANCE OF HOLSTEIN CALVES IN AN ACCELERATED GROWTH SYSTEM

6.1 Introduction

The amount of milk consumed by young dairy calves influences gut development (Anderson et al., 1987) and determines intake of starter feed as well as health and growth of calves (Appleby et al., 2001). Studies to improve milk feeding systems for dairy calves through *ad libitum* milk feeding have shown higher milk consumption and BW gain, with reduced starter intake, compared to restricted milk feeding (Appleby et al., 2001; Hammon et al., 2002; Jasper and Weary, 2002). Delayed solid feed intake because of *ad libitum* milk consumption during the pre-weaning period (Appleby et al., 2001; Hammon et al., 2002) results in delayed ruminal development which is associated with poor post-weaning performance (Baldwin et al., 2004) and poor welfare (Khan et al., 2007a).

Rumen development is an important factor determining early solid feed intake and performance in cattle (Gorka et al., 2009). Butyrate and propionate are the primary VFA's responsible for rumen epithelial and papillae development in the pre-weaned ruminant (Coverdale et al., 2004). *Megasphaera elsdenii* is thought to play a major role in production of branched-chain volatile fatty acid (VFAs) in the rumen (Wallace 1986) and may ferment to favour propionic and/or butyric acid as end product at the expense of acetic acid (Henning et al., 2010b).

Dosing *M. elsdenii* NCIMB 41125 to calves on d 7 and d 14 resulted in improved starter DMI, with calves dosed on d 14 consuming numerically more calf starter DM than calves dosed on d 7. (Experiment 2). It is possible that an organism such as *M. elsdenii* can play a positive role in rumen development under condition where starter intake is suppressed due to *ad lib* or high volume milk feeding in accelerated growth feeding systems. The objective of this study was to evaluate the influence of an oral dose of *M. elsdenii* NCIMB 41125 at 14 d of age on pre- and post-weaning intake, performance, and ruminal development of calves fed milk *ad lib* during the two feeding periods in the pre-weaning phase. This feeding regime can be regarded as an accelerated growth feeding system.

6.2. Materials and methods

6.2.1. Animals and treatments

The experimental protocol and procedures were approved by the Animal Ethics Committee (APIEC11/028) of the Agricultural Research Council at Irene, Pretoria, South Africa. Twenty six Holstein calves (BW = 34.5 ± 1.65 kg) from the Agricultural Research Council Dairy herd were blocked on the basis of order of birth and sex and randomly assigned at birth to 2 treatments [males (n = 6), females (n = 7) per treatment] and fed for until two weeks after weaning at 56 day. Treatments were a control group, which did not receive *M. elsdenii* (Me0) and a *M. elsdenii* group, which received a 50-mL oral dose of *M. elsdenii* NCIMB 41125 (10^8 CFU/mL) at 14 d of age (Me14). Calves were fed colostrum for the first 3 d of life followed by free choice access to whole milk (CP: 3.27 %; Fat: 3.55 %; Lactose: 4.75 %) during feedings at 8:00 and 14:00. Milk was offered in a 5 L bucket and if the calf consumed all of the 5 L it was immediately refilled until voluntary intake ceased. From d 52 until weaning (d 56) milk intake was limited to 4 L/d, offered once daily at 8:00. A commercial calf starter (Table 6.1) was offered *ad libitum* starting at 4 d of age until the end of the study. Fresh water was available *ad lib* throughout the study. Intake of whole milk and calf starter feed were measured daily and body weights were taken weekly.

6.2.2. Measurements and sample collection

Daily calf starter, milk and water consumption by each calf were measured throughout the experiment. Starter was offered at 08h00, with starter intake recorded daily for each calf. Calves were initially offered 250 g of starter, and remaining feed was weighed back at each delivery time. Starter increased with 250 g increments when calves refused less than 50 g of feed. Water was offered *ad libitum*, beginning on d 4.

Table 6.1. Chemical composition of calf starter pellets¹ fed to calves dosed or not with *M. elsdenii* NCIMB 41125

DM, g/kg	892
CP, g/kg DM	191
Fat, g/kg DM	39
NDF, g/kg DM	239
ME, MJ/kg of DM ²	14.5
Ca, g/kg	8.0
P, g/kg	5.5

¹Contained a premix that supplied per kg: 7 500 IU vitamin A; 1 020 IU vitamin D3; 27 mg vitamin E; 0.5 mg Co; 15 mg Cu; 1 mg I; 30 mg Fe; 60 mg Mn; 0.24 mg Se; 70 mg Zn.

²Metabolizable energy calculated according to NRC (2001).

The dry matter, crude protein, and fat contents were determined according to the standard AOAC procedures (2000). Calcium (Ca), potassium (K) and magnesium (Mg) were determined according to Giron, (1973) using a Perkin Elmer Atomic Spectrophotometer. Phosphorus (P) was assayed according to AOAC (2000) procedure 965.17. The NDF was analyzed using the methods of VanSoest et al. (1991) with an ANKOM fiber analyzer.

Calves were weighed at birth and at 7 day intervals throughout the experiment before AM milk and starter feeding. Average daily gain was calculated from weekly weight gain. Average BW gain, total DM intake, gain:feed (kg BW gain/kg of total DM intake) and ME conversion ratio (kg BW gain/kg of total ME intake) were calculated. Metabolisable energy concentration of whole milk was calculated using NRC (2001) equations as $ME (MJ/kg) = 3.77 ((0.057 \times CP \%) + (0.092 \times Fat \%) + (0.0395 \times Lactose \%))$ and predicted ADG from individual birth weight was calculated based on equations of energy requirements for calves fed milk (NRC, 2001). At 7, 21, 28, 42, and 56 d of age post-feeding, blood was collected via the jugular vein from selected calves in both groups (8 per group) by venipuncture for analysis of BHBA. Blood was collected in 10 mL collection tubes containing sodium heparin. Two ml of blood were then transferred to two clean test tubes (in duplicate). Cold 30% perchloric acid was added in a 1:1 ratio to the blood samples, for the precipitation of protein. After thorough mixing, the precipitated protein was removed by centrifuging in a refrigerated centrifuge at 2000 rpm, for 20 min. The clear supernatant was transferred to clean glass tubes and recapped with clean screw caps, as quickly as possible to prevent evaporation of acetone, and stored at $-20^{\circ}C$, until analyses for BHBA. The BHBA was analysed by means of an enzymatic analysis (Williamson et al., 1962). At weaning, calves in both groups continued on the starter feed and remained in individual pens until 70 d of age when the experiment concluded.

6.2.3. Statistical analysis

Data were analyzed as repeated measures for two periods (pre- and post-weaning) using the PROC MIXED model of SAS (SAS Institute, 2009). Parameters were pooled by week for analysis. The statistical model included calf as a random effect, and experimental group and its interaction with time as a fixed effect. The model was subjected to an autoregressive order one. The statistical model used was

$$Y_{cgt} = \mu + ag + \beta t + (\alpha\beta)gt + \gamma(\alpha)cg + ecgt,$$

where Y_{cgt} = an observation value for parameters measured from calf c from group g at time t ;

μ = overall mean for the population;

ag = fixed effect of group g , where g = group Me0 or Me14;

βt = fixed effect of time t

$(\alpha\beta)gt$ = fixed interaction of effect of group g and time t ;

$\gamma(\alpha)cg$ = random effect of calf c nested within group g ; and

$ecgt$ = error associated with the measurement taken from calf c from group g at time t .

Significance was declared at $P < 0.05$ and tendencies at $P < 0.10$. A linear mixed-effects model was also performed to compare predicted and observed ADG within groups.

6.3. Results and discussion

6.3.1. Intake, growth and efficiency

Least squares means for average daily starter intake, milk intake, ME intake, starter and total DMI, gain:feed ratio, BW and ADG for both the pre-weaning period and two weeks after weaning are presented in Table 6.2. All calves had free access to milk during feeding time and consumed similar amounts of milk ($P > 0.05$) averaging 7.8 kg/d, which was comparable to 8.1 kg/d reported by Borderas et al. (2009) for calves fed *ad lib* through an automated milk feeder. This level of milk approached 8.79 and 8.97 kg/d reported by Jasper and Weary (2002) and Moallem et al. (2010), respectively, for calves fed whole milk *ad lib*.

All calves had low starter DMI during pre-weaning due to increased milk supply and associated increase in nutrient availability (Appleby et al., 2001; Cowles et al., 2006; Hill et al., 2008b). Dosed calves consumed more ($P < 0.05$) starter DMI than Me0 calves, but total DMI (calf starter + milk) and estimated ME intake were not different between treatments.

Mean starter DMI for control calves (0.10 kg/d) was similar to 0.09 kg/d reported by Jasper and Weary (2002) for calves fed whole milk *ad lib*. Dosed calves approached 0.17 kg/d for calves fed *ad lib* milk for weaning at 60 d observed by Moallem et al. (2010). The calves in the latter study were heavier (41 vs 34.5 kg birth weight) than calves used in the present study. Dosed calves consumed an average of 0.14 kg/d starter DM during the pre-weaning period, which represented 40.0 % more starter feed than control calves, which may be attributed to more developed rumen papillae and associated increased absorption capacity of nutrients allowing high intake of feed during time when milk was not available. These calves reached 0.225 kg/d of starter DMI during week 6 (Table 6.3) when milk intake was 9 kg/d. The more developed calf rumen observed in conventional rearing systems compared to accelerate growth, is explained by higher ruminal butyrate concentration provided by concentrate fermentation (Heinrichs and Lesmeister, 2005).

A different progressive change of starter intake over time in favour of Me14 calves during the pre-weaning period was indicated by the significant ($P = 0.005$) interaction between treatment and time (Table 6.2).

Calf starter DMI was also compared within week (Table 6.3) and before weaning the treatment difference occurred during week 7 and 8 where dosing *M. elsdenii* NCIMB 41125 increased DMI when compared to control calves ($P < 0.05$). After weaning, dosed calves tended ($P = 0.07$) to consume more and consumed more ($P = 0.01$) starter DM during week 9 and 10, respectively, when compared to control calves. Pre-weaning average daily gain was only numerically higher in Me14 group compared to Me0, but Me14 calves were 5.8 kg heavier at weaning compared to Me0 calves. Gain:feed was greater for Me14 calves compared to Me0 calves ($P < 0.0001$). Post-weaning DMI and estimated ME intake were greater ($P < 0.05$) for Me14 calves compared to Me0 calves and Me14 calves gained 0.37 kg more per day than Me0 ($P = 0.02$) calves and weighed 11.6 kg more than the Me0 calves at the end of the study.

Table 6.2. Least square means of intake, growth and efficiency of calves dosed (Me14) or not (Me0) with *M. elsdenii* NCIMB 41125.

	Treatment*		SEM ¹	P-value**		
	Me0	Me14		T	Time	T x Time
Prewaning						
Milk intake, kg/d	8.2	7.4	0.211	0.010	<0.0001	0.4437
Starter DMI, kg/d	0.10	0.14	0.011	0.001	<0.0001	0.0048
² Tot DMI, kg/d	1.12	1.06	0.026	0.358	<0.0001	0.7116
³ ME intake, MJ/d	24.0	22.1	0.134	0.143	<0.0001	0.6634
Initial BW, kg	34.3	34.8	1.654	0.820	-	-
Weaning BW, kg	69.7	75.5	2.320	0.012	-	-
⁴ ADG, kg	0.63	0.73	0.048	0.261	<0.0001	0.5719
Gain:Feed, kg DM/kg gain	0.57	0.69	0.023	0.029	<0.0001	0.2955
Postweaning period						
Starter DMI, kg/d	1.46	1.73	0.065	0.027	<0.0001	0.594
ME intake, MJ/d	21.1	25.0	0.310	0.023	<0.0001	0.594
Final BW, kg	77.4	89.9	2.012	<0.0001	-	-
ADG, kg	0.55	1.03	0.123	0.002	0.048	0.2613
Gain:Feed	0.37	0.60	0.120	0.068	0.6060	0.1958

*Me0: control group, which did not receive *M. elsdenii*; Me14: received a 50-mL oral dose of *M. elsdenii* NCIMB 41125 (10^8 CFU/mL) at 14 d of age

**T: Effect of treatment (dosing or not with *M. elsdenii*); T x Time (week): Interaction between treatment and time

¹SEM: Standard error of mean

²Tot DMI: Starter DM + Milk DM

³ME intake: (Starter ME x Starter DMI) + (Milk ME x Milk intake)

⁴ADG: Average daily gain calculated from weekly ADG.

The progressive change in milk and starter DMI over time are shown in Figures 6.1 and 6.2 respectively. In agreement with previous studies, all calves increased milk consumption during the first 2 weeks and were able to consume a large quantity of milk (de Passille´ et al. 1992; Khan et al., 2006). There was fluctuation of milk intake throughout the pre-weaning period in both groups, with the average milk intake increasing with age. Starter DMI was very low in both treatments, but exponentially increased from day 52 to 70. This was related to milk withdrawal, as it was reduced to 4 L once in the morning until weaning at 56 days. Differences between treatments in starter feed intake became apparent from week 7.

The average daily weight gains did not differ ($P < 0.05$) between treatments during the pre-weaning period, but was higher ($P < 0.05$) in Me14 group during the post-weaning.

Table 6.3. Least square mean of weekly starter dry matter intake of calves dosed (Me14) or not (Me0) with *M. elsdenii* NCIMB 41125.

	Treatment*		SEM ⁱ	P-value
	Me0	Me14		
Week 1	0.017	0.013	0.0029	0.915
Week 2	0.033	0.030	0.0460	0.937
Week 3	0.056	0.061	0.0063	0.874
Week 4	0.066	0.071	0.0069	0.903
Week 5	0.079	0.098	0.0098	0.526
Week 6	0.104	0.137	0.0104	0.161
Week 7	0.117	0.225	0.0234	0.002
Week 8	0.354	0.513	0.0456	<0.001
Week 9	1.150	1.371	0.0624	0.071
Week 10	1.781	2.092	0.0668	0.012

*Me0: control group, which did not receive *M. elsdenii*; Me14: received a 50-mL oral dose of *M. elsdenii* NCIMB 41125 (10^8 CFU/mL) at 14 d of age

The ADG of Me0 calves increased ($P < 0.05$) from d 21 to d 42 of the study from 0.51 to 1.00 kg/d (Figure 6.3), with a decline to 0.46 kg/d on d 35, while it increased ($P < 0.05$) during the same period from 0.51 to 1.2 kg/d in Me14 group without declining. In both groups ADG decreased at weaning to less than 0.4 kg/d, but remained numerically higher in Me14 calves. After weaning, the ADG of calves increased ($P < 0.05$) within and between treatments giving a net growth advantage to Me14 calves.

Pre-weaning gain:feed was lower ($P < 0.05$) for Me0 compared to Me14, but tended ($P = 0.07$) to be higher for Me14 post-weaning compared to Me0.

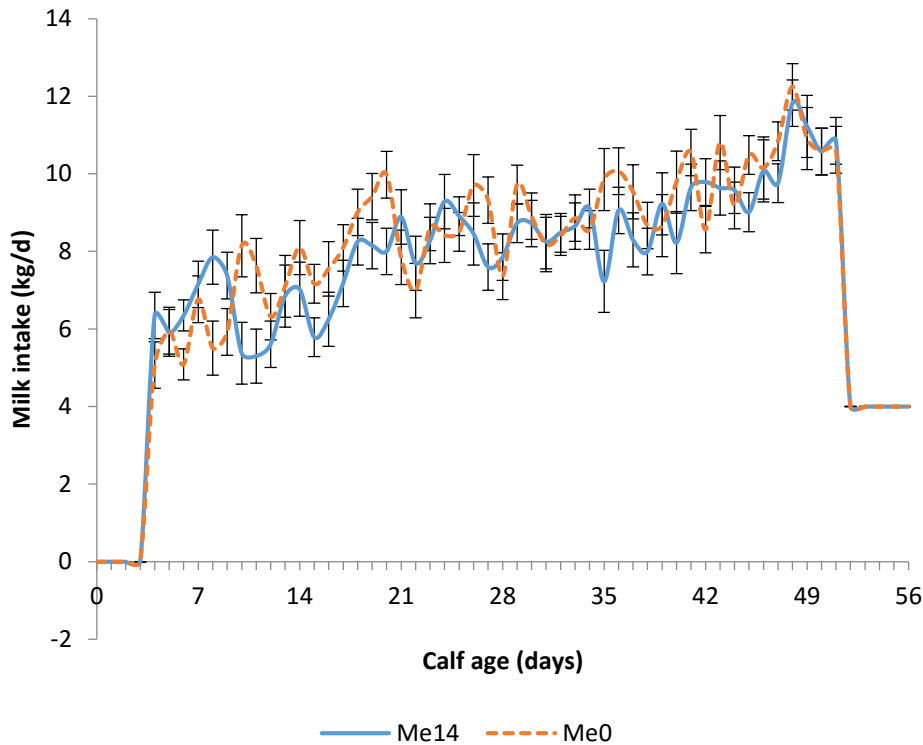


Figure 6.1. Milk intake (\pm SE) over period for calves dosed (Me14) or not (No Me) with *M. elsdenii* NCIMB 41125 on day 14 of age.

The decrease in ADG around weaning reported in previous studies (Bar-Peled et al., 1997; Jasper and Weary, 2002; Cowles et al., 2006; Terré et al., 2006) in calves fed high volumes of milk was also found in the current study, as all calves decreased ADG a week before weaning. This is explained by the decrease in energy and protein intake when milk was reduced. However, Me0 calves presented a more pronounced decrease compared with Me14 calves. A common pitfall encountered in high milk feeding programs is the low starter intake at weaning (Bar-Peled et al., 1997; Brown et al., 2005; Cowles et al., 2006). Unlike, increased starter feed in the Me14 group gave them a growth advantage after weaning as reflected in the high post-weaning ADG, suggesting improved intake of energy when calves were weaned from milk, as starter was the only source of energy available. This suggests that dosing *M. elsdenii* could be an efficient strategy for weaning calves adequately when fed high volumes milk or milk replacer. This may be attributed

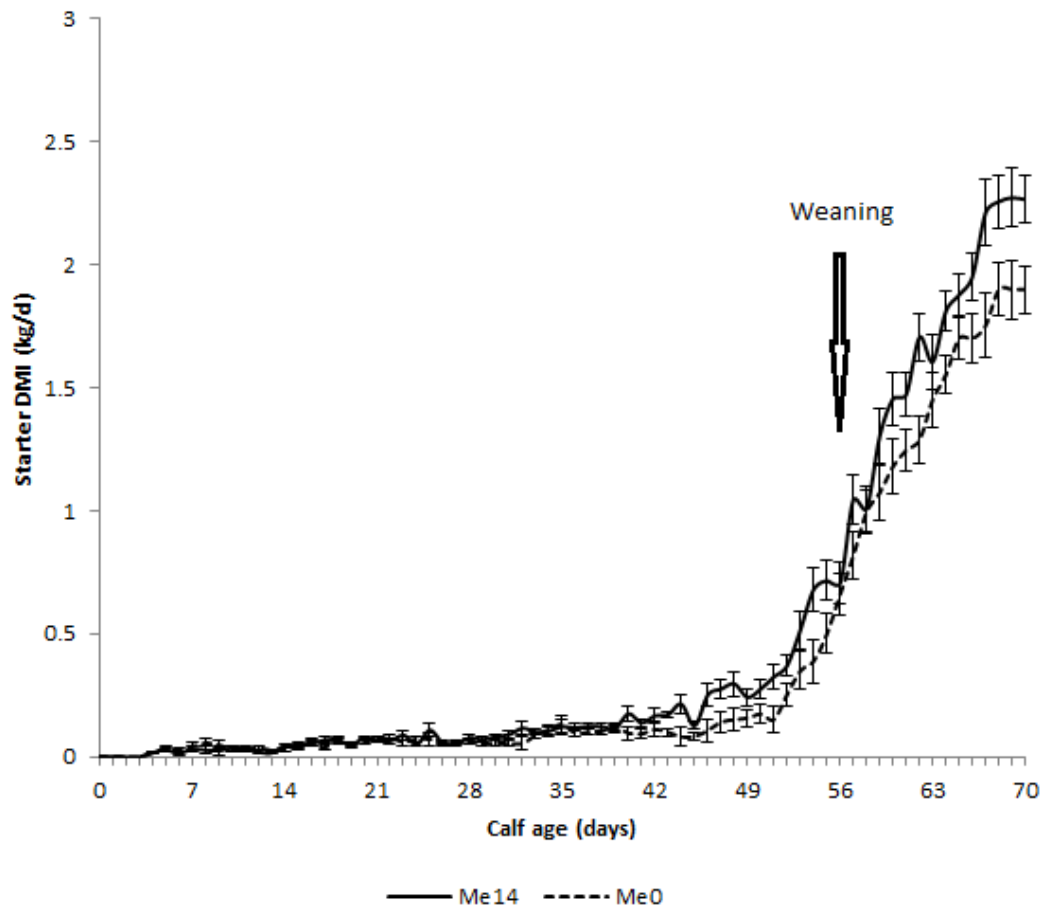


Figure 6.2. Starter dry matter intake (\pm SE) over period for calves dosed (Plus Me) or not (No Me) with *M. elsdenii* NCIMB 41125 on day 14 of age.

to a residual effect of early increased starter DMI and, therefore, increased gut fill. Early starter consumption increases nutrient digestibility, and consequently improves ruminal microbiota and fermentation activities (Anderson et al., 1987).

In a previously reported study (Fathi et al., 2009), a 40 g/d increase in starter DMI (similar to the current study) due to feeding vanilla flavoured calf starter during the pre-weaning period, resulted in calves gaining on average 70 g/d and being 4 kg heavier than calves fed unflavoured starter at weaning at 42 d. In the current study, dosed calves gained 100 g/d and were 5 kg heavier than control calves at weaning at 56 d. The higher starter DMI was more pronounced during the last two weeks prior weaning, and was 108 and 159 g/d during week 7 and 8, respectively, when the milk intake was similar between the two groups. The improved solid feed consumption of calves dosed with *M. elsdenii*, and possible, related ruminal activity have

mitigated negative effects of ad libitum milk intake and cushioned them from transitional effects of milk removal on growth and performance.

The average daily gain of pre-weaned calves was compared with predicted ADG (NRC 2001) within groups to ascertain the efficiency of nutrient utilization, and results are presented in Figure 6.4. In the Me14 group, observed ADG was higher ($P < 0.05$) than predicted ADG, while in the Me0 group observed ADG did not differ from the model.

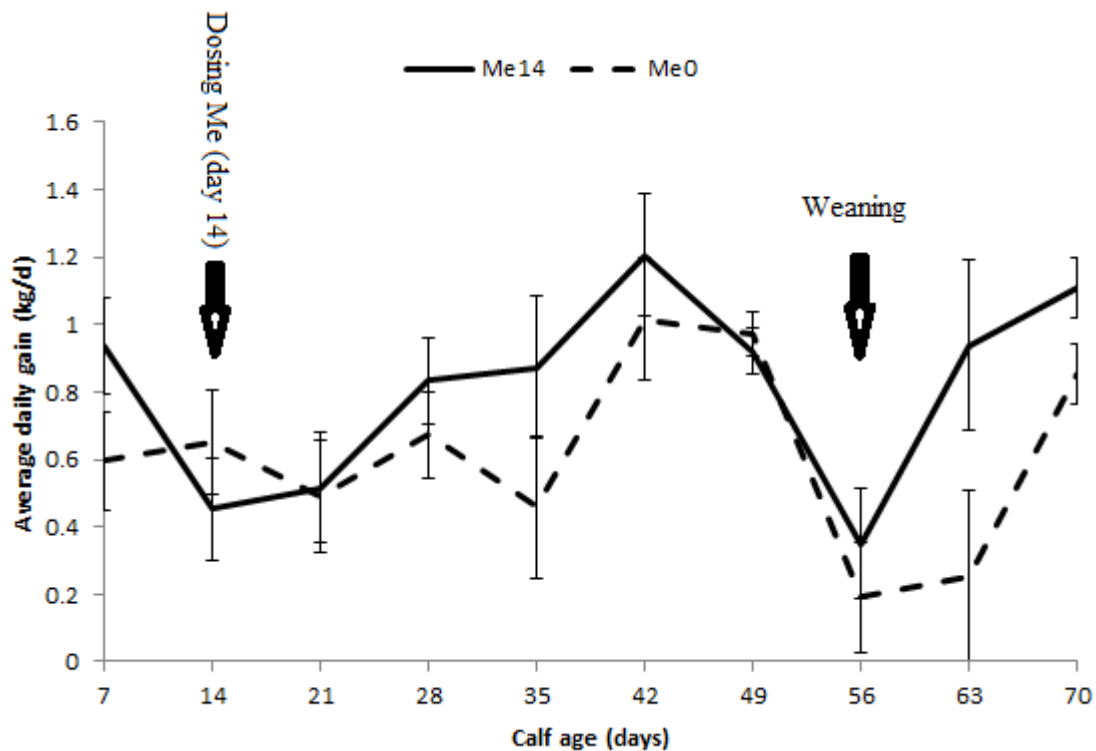


Figure 6.3. Average daily gain (\pm SE) over the experimental period for calves dosed (Me) or not (Cont) with *M. elsdenii*

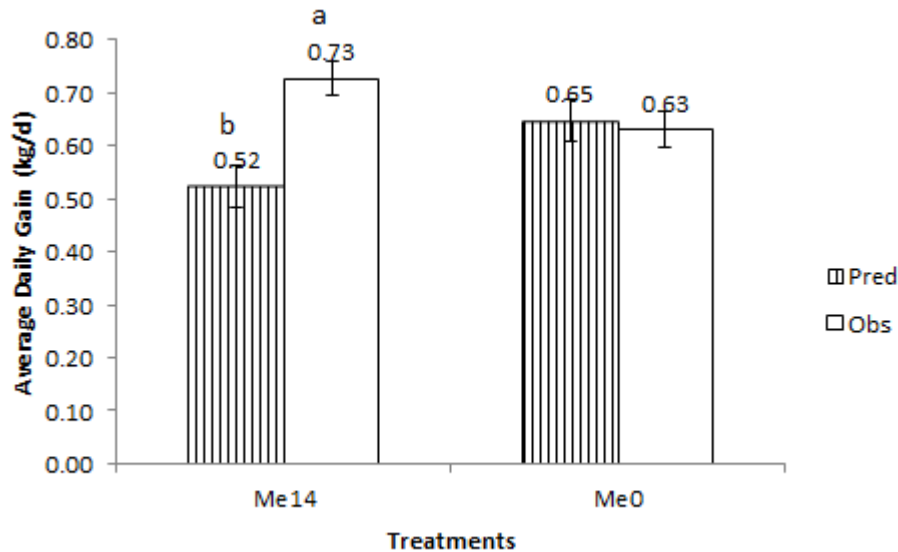


Figure 6.4. Comparison of observed and predicted average daily gain, using equation based on metabolisable energy requirement (NRC, 2001) during the pre-weaning period of calves dosed (Me14) or not (Me0) with *M. elsdenii*.

^{ab}Least square means differ significantly between treatment groups at the same time point ($P < 0.01$).

Higher actual pre-weaned ADG observed in the Me14 group than predicted by the NRC (2001) model may indicate more efficient utilization of starter nutrients by calves that received *M. elsdenii*, supporting high ME intake and weaning BW observed in this group. This is related to improved starter DMI observed in the Me14-calves. They consumed 40% more starter than Me0 calves, and therefore were more adapted to dry feed intake, probably due to an improved absorptive capacity (Coverdale et al., 2004), which is believed to be the result of more butyric acid produced in the rumen. Butyric acid provides energy for thickening of the rumen wall, formation of papillae, and increasing capillary development (Weigand et al., 1975).

6.3.2. Blood beta-hydroxybutyrate

Results of effects on Plasma BHBA concentration are presented in Figure 6.5. On d 7 (prior to treatment administration) BHBA concentrations tended ($P < 0.10$) to be higher for Me0 calves compared to Me14 calves, however BHBA concentration drastically increased in Me14 from d 21 and until d 56 ($P < 0.05$).

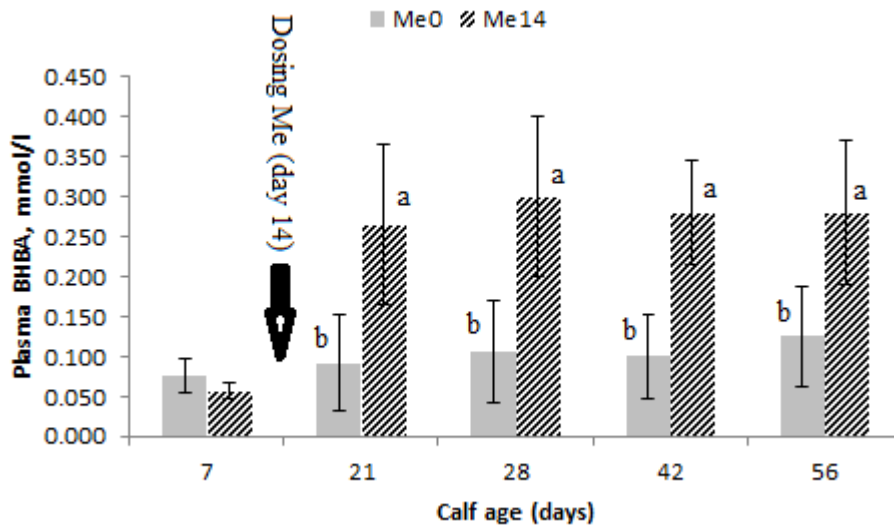


Figure 6.5. Plasma beta-hydroxybutyrate (BHBA) of calves dosed (Me14) or not (Me0) with *M. elsdenii*.^{ab}Least square means differ significantly between treatment groups at the same time point ($P < 0.01$).

The potential of *M. elsdenii* to increase the proportion of rumen propionate and/or butyrate was previously reported (Aikman, 2008; Aikman et al., 2009; Marounek et al., 1989; Cruz et al., 2001; Henning et al., 2010b), and the particular role of these two VFA's on stimulating rumen epithelial cells and papillae development and thereby, increasing the capacity of solid feed intake has been previously discussed (Tamate et al., 1962; Coverdale et al., 2004; Lane and Jesse, 1997). Recently, Guilloteau et al. (2009) and Ślusarczyk et al. (2010) observed improved growth performance of calves with the addition of sodium butyrate to milk. Supplementation with 3 % Na-butyrate in milk for 56 days, improved growth and feed conversion of pre-weaned calves, but decrease feed intake due to increased odour offensiveness of the diets. The effects of VFA's are reported to be most likely associated with the rate at which they are metabolized by mucosal cells during absorption, being over 90% of butyric acid and approximately 50% of propionic acid metabolized and oxidized to ketone bodies (Britton and Krehbill, 1993). As it is oxidized by the rumen epithelial cells and passes through the rumen wall, butyric acid is converted to β -hydroxybutyrate, therefore constituting a measurement of rumen epithelial metabolism and indicator of rumen development (Quigley, 1991; Lesmeister and Heinrichs, 2004). In the present study, these effects are supported by results on BHBA, an important metabolite used by the body as an energy source. Similar to previous findings (Quigley and Bernard, 1996; Ślusarczyk et al., 2010), calves in the present study increased blood BHBA with age, but this increase was more pronounced in Me14-calves. These calves had increased plasma BHBA levels the week after being dosed with *M. elsdenii* NCIMB 41125, consumed

more calf starters than control calves from 2 week before weaning until the end of the experimental period and maintained their growth advantage after weaning. Ślusarczyk et al. (2010) did not observe any change in plasma BHBA with the addition of Na-butyrate due to the observed decrease of feed intake caused by the odour.

6.4. Conclusion

Administering *Megasphaera elsdenii* NCIMB 41125 to Holstein calves at 14 d of age in an accelerated growth program improved pre-and post-weaning performance, starter feed intake and feed efficiency. Improvements in starter feed intake suggest higher ruminal VFA concentrations to stimulate rumen development. In addition higher blood beta-hydroxybutyrate concentrations may indicate greater metabolic activity of the rumen epithelium for calves dosed with *M. elsdenii* NCIMB 41125.

CHAPTER 7

RUMEN DEVELOPMENT, RUMINAL FERMENTATION AND BLOOD B-HYDROXYBUTYRATE SYNTHESIS IN PRE-WEANED DAIRY CALVES DOSED WITH *MEGASPHAERA ELSDENII* NCIMB 41125.

7.1. Introduction

Many dairy producers wean their calves from whole milk or milk replacer early at a relatively young age (3 to 6 weeks) (Kehoe et al., 2007). Rapid rumen development is therefore critical in early weaning systems as it impacts on post weaning intake of solid feed, growth performance and health of calves (Greenwood et al., 1997; Baldwin et al., 2004). Rumen development in calves is dependent on intake of solid feed (Lesmeister and Heinrichs, 2004), which is fermented to volatile fatty acids that stimulate rumen papillae development (Sander et al., 1959). Starter feeds that promote production of VFA's such as butyrate are the preferred type of calf starter as it triggers papillae growth in the rumen (Stobo et al., 1966). *Megasphaera elsdenii*, a gram negative bacteria, plays a major role in the production of branched-chain VFA's in the rumen (Wallace 1986). This bacteria converts lactic acid to propionate and butyrate and converts glucose to butyrate (Marounek et al., 1989; Henning et al., 2010b). The potential of *M. elsdenii* to increase molar proportion of rumen propionate and/or butyrate has been documented (Marounek et al., 1989; Cruz et al., 2001; Aikman, 2008; Aikman et al., 2009; Henning et al., 2010b) and the role of these two VFA's on stimulating rumen epithelial cells and papillae development and their role in increasing the capacity of solid feed intake were reviewed (Warner et al., 1956; Tamate et al., 1962; Lane and Jesse, 1997; Coverdale et al., 2004). Butyric acid provides energy for thickening of the rumen wall, formation of papillae, and increasing capillary development (Weigand et al., 1975). Dosing calves with *M. elsdenii*, therefore, could have the potential to increase production of butyric acid and thereby accelerate rumen development during the pre-weaning phase. The objectives of this experiment were to determine the effects of dosing calves with *M. elsdenii* on solid feed intake, growth performance, serum levels of beta-hydroxybutyric acid (BHBA) and rumen development of calves.

7.2. Materials and methods

7.2.1. Animal and diets

The experimental protocol and procedures were approved by the Animal Ethics Committee (APIEC11/028) of the Agricultural Research Council at Irene, Pretoria, South Africa.

Thirty healthy Holstein calves (34.6 ± 5.04 kg BW) from the Agricultural Research Council/Animal Production Institute in Pretoria (South Africa) were blocked on the basis of order of birth and sex and randomly allocated to one of two treatment groups [males ($n = 7$), females ($n = 8$) per treatment]. Treatments were a control group (Me0), which did not receive *M. elsdenii* and Me14, which received a 50-mL oral dose of *M. elsdenii* NCIMB 41125 (10^8 CFU/mL) at 14 d of age. Calves were given colostrum for the first 3 d of life before receiving a commercial calf starter (CP: 191 g/kg DM; Fat: 39 g/kg DM; NDF: 239 g/kg DM; ME:14.5 MJ/kg DM; Ca: 8.0 g/kg DM; P: 5.5 g/kg DM) offered *ad libitum* starting at d 4 of age until weaning at d 42. The milk feeding regime is shown in Table 7.1 and fresh water was available throughout the study.

Table 7.1. Milk¹ feeding regime of calves dosed or not with *M. elsdenii* NCIMB 41125

		Morning	Afternoon
Day 4 to day 7	Milk	2 litres	2 litres
Day 8 to day 14	Milk	3 litres	3 litres
Day 15 to day 35	Milk	2 litres	2 litres
From Day 36 to weaning	Milk	2 litres	-

¹ Average milk composition: CP: 3.21 %; fat: 3.51 %; lactose: 4.72 %

Calves received 2 L of whole milk in the morning (8:00) and 2 L in the afternoon (14:00) from d 4 to d 7 before being switched to 3 L in the morning and 3 L in the afternoon from d 8 to d 14. The milk feeding was thereafter reduced to 2 L at 8:00 and 2 L at 14:00 from d 15 to d 35, thereafter calves received 4 L once at 8:00 until weaning on d 42.

7.2.2 Measurements and sample collection

Consumption of starter, milk and water by each calf were measured daily throughout the experiment to estimate total DM intake. Calves were initially offered 250 g of starter, and remaining feed was weighed back at each feeding time the next day in order to determine calf starter intake. Daily starter feed offerings were changed in 250 g increments when calves refused less than 50 g of feed. Calves were weighed at birth and at 7 day intervals throughout the experiment. weighing was done before AM milk and starter feeding. Average daily gain (ADG) was calculated from weekly weight gain. Seven bull calves were selected from each treatment for blood collection at 7, 14, 21, 28, and 42 d of age post-feeding via the jugular vein by venipuncture for determination of BHBA. Blood was collected into 10 mL collection tubes containing sodium heparin; two mL of blood were then transferred to two clean test tubes (in duplicate). Cold 30% perchloric acid was added in a 1:1 ratio to the blood samples, for the precipitation of protein. After thorough mixing, the precipitated protein was removed by centrifuging in a refrigerated centrifuge at 2000 rpm, for 20 min. The clear supernatant was quickly transferred to clean glass tubes and recapped with clean screw caps, to prevent evaporation of acetone, and stored at -20°C , until analyses for BHBA. The determination of BHBA was carried out by means of an enzymatic analysis (Williamson et al., 1962).

Fourteen male calves (7 per group) were euthanised on d 42 using captive bolt stunning for determination of VFA's and rumen development. A sample of rumen fluid was collected immediately for analysis of VFA's. Four mL of the filtered rumen digesta was preserved with 1 ml of 25% H_3PO_4 (orthophosphoric acid) and then stored at -10°C for VFA analysis using a gas chromatography (Gibbs *et al.*, 1973).

Measurements for papillae length, papillae width, and rumen wall thickness were performed according to Lesmeister et al. (2004b). The rumen was harvested (Figure 7.1), emptied, and rinsed with cold water. Rumen tissue samples were collected from the cranial and caudal sacs of the ventral and dorsal portions of the rumen (area = 4 cm^2). Three random tissue samples (1-cm^2 each) were collected from each area ($n = 12$) and duplicate measurements for papillae length, papillae width, and rumen wall thickness were recorded ($n = 24$).



Figure 7.1. Rumen harvested from a 42 d old calf.

Tissue samples were fixed in a 30% formaldehyde solution for subsequent measurements. Visual measurements were taken using a Bausch & Lomb Stereo Zoom microscope (Rochester 2, New York, United State) fitted with a measuring eyepiece. Data from all 14 calves were pooled for procedure analysis.

7.2.3. Statistical analysis

Average daily gain, calf starter and total DMI, and plasma BHBA were analyzed as repeated measures using the PROC MIXED statement of SAS (SAS Institute, 2009). Parameters measured daily were pooled by week for analysis. The statistical model included calf as a random effect, and experimental group and its interaction with time as a fixed effect (Littell et al., 1998). The model was subjected to an autoregressive order one. The statistical model used for repeated measure analyses was

$$Y_{cit} = \mu + \alpha_i + \beta_t + T_{it} + \delta_{ci} + e_{cit},$$

where Y_{cit} = an observation value for ADG, starter DMI, total DMI and plasma BHBA measured from calf c from treatment i at time t ;

μ = overall mean for the population;

α_i = fixed effect of treatment i , where i = Me0 or Me14;

β_t = fixed effect of time t , where $t = w$ 1, 2, 3, 4, 5 or 6 for starter DMI, total DMI, ADG and BHBA;

T_{it} = fixed interaction of effect of treatment i and time t ;

δ_{ci} = random effect of calf c nested within i treatment; and

e_{cit} = error associated with the measurement taken from calf c from group g at time t .

Means for gain:feed, initial and weaning BW, predicted ADG as well as stomach development parameters, were subjected to ANOVA using PROC GLM (SAS Institute, 2009). The statistical model used was

$$Y_{ci} = \mu + T_i + \delta_c + e_{ci}$$

Where Y_{ci} = observation value for Body weight, ADG, starter DMI, total DMI, and rumen development taken from calf c at t time.

μ = overall mean of the population,

T_i = fixed effect of the i^{th} treatment (Me0 or Me14),

δ_c = random effect of calf, and

e_{ci} = error associated with the measurement taken from calf c from i^{th} treatment

Significance was declared at $P < 0.05$ and tendencies at $P < 0.10$.

7.3. Results and discussion

7.3.1. Intake, body weight, body weight gain and plasma BHBA

In Table 7.2 is shown the least squares means for calf starter and total DMI, BW, ADG, and gain:feed ratio. Calves in the Me14 group consumed more starter feed and total dry matter (milk + starter) than Me0 calves ($P < 0.05$) and tended to gain 17% more in ADG than Me0 ($P = 0.10$). Increased feed intake was previously reported when lambs (Henning et al., 2010b) and steers (Henning et al., 2010) were drenched with *M. elsdenii* NCIMB 41125. No difference in DMI was found in feedlot steers fed high and low roughage diets (Leeuw et. al., 2009), although the ADG increased from week 3 after dosing with *M. elsdenii*. In another study using high risk calves (Miller et al., 2013), administration *M. elsdenii* NCIMB 41125 induced an 8 and 25 % increase in DMI and ADG, respectively. The stimulating effect on feed intake was also

observed in adult animals by Drouillard (2004), who found that cattle drenched with *M. elsdenii* NCIMB 41125 tended to maintain higher intakes throughout the experiment than control cattle.

Weaning BW, was also higher for calves dosed with Me14 compared to Me0 ($P = 0.03$). The gain:feed ratio was not different between treatments ($P > 0.05$).

The 29.7 % increase in starter DMI by dosed calves compared to Me0 calves, suggests a more developed absorptive capacity (Coverdale et al., 2004). A higher intake of calf starter is important in early weaning management systems because it impacts on general calf growth performance and calf health post weaning (Greenwood et al., 1997). In pre-weaned calves, rumen development in turn plays a major role in intake of solid feed (Kristensen et al., 2007) and affects digestion and absorption of nutrients (Gorka et al., 2011).

Administering *M. elsdenii* NCIMB 41125 tended to increase ADG ($P < 0.10$) by 17%, resulting in a 5.3 kg heavier final weight compared to control calves. This can be attributed to increased nutrient availability due to early increased starter DMI, and consequently improved ruminal fermentation activities. Miller et al (2013) also reported that high risk crossbred steers were 10 kg heavier than control steers after 64 days feeding period.

A different evolution of starter intake throughout the study was indicated by a significant ($P < 0.001$) interaction between treatment and time. The starter DM intake was similar ($P = 0.51$) averaging 0.11 ± 0.03 kg/d during the first 14 d of study but greater ($P < 0.05$) in Me14 compared to Me0 afterwards (Figure 7.2). However, as was the case with study 2, the significant increase in calf starter observed in Me14 calves did not translate into higher gain:feed ratio as it did not differ between Me0 and Me14, averaging 0.50. Previous studies (Barlet 2001; Brown et al., 2005; Khan et al., 2007) reported gain:feed ratios of 0.55, 0.44 and 0.40, respectively for calves fed milk in a conventional growth system.

Table 7.2. Effect of dosing calves with *M. elsdenii* NCIMB 41125 on their growth performance, feed intake and blood BHBA concentrations.

Items	Treatments*		SEM ¹	P-Value		
	Me0	Me14		T	Time	T x Time
BW, kg						
Initial	33.4	35.8	1.7	0.18	-	-
Weaning	50.5	55.8	2.2	0.03	-	-
² ADG, kg/d	0.41	0.48	0.05	0.10	0.018	0.20
Starter DMI, kg/d	0.37	0.48	0.01	<0.001	<0.001	<0.001
³ Total DMI, kg/d	0.89	1.00	0.01	<0.001	<0.001	<0.001
⁴ Gain: Feed, kg/kg	0.46	0.48	0.04	0.68	-	-
Plasma BHBA, mmol/l	0.17	0.22	0.01	<0.003	<0.001	0.23

Values in the same row differ if $P \leq 0.05$ or tend to differ if $P < 0.10$

*Me0: control group, which did not receive *M. elsdenii*; Me14: received a 50-mL oral dose of *M. elsdenii*

NCIMB 41125 (10^8 CFU/mL) at 14 d of age

T: Effect of treatment (dosing or not with *M. elsdenii*); T x Time (week): Interaction between treatment and time

¹SEM: Standard error of mean

²ADG: Average daily gain calculated from weekly ADG.

³Tot DMI: Starter DM + Milk DM

⁴Gain: Feed: Calculated as body weight gain / Starter DMI

The change in plasma BHBA concentration is shown in Figure 7.3. Plasma BHBA was higher ($P = 0.003$) in dosed calves compared to control calves. As for starter DMI, there was a significant interaction between treatment and time for BHBA concentrate. Plasma BHBA concentration increased with age in both groups, but the increase was minimal from d 7 to d 14 and did not differ between Me0 and Me14 groups averaging 0.10 and 0.11mmol/l, respectively ($P > 0.10$). On d 21 BHBA increased rapidly in both groups and averaged 0.17 mmol/l. From d 28 to d 42, plasma BHBA in Me14 calves were higher than in Me0 calves with concentration of 0.22 and 0.17 mmol/l, respectively ($P < 0.05$). After 42 days, the trend was still for plasma BHBA to increase in Me14 calves, while it tended to decline in Me0 calves after day 35.

Similar to plasma BHBA concentration, its precursor rumen butyrate concentration was found to be higher in Me14 calves ($P < 0.05$) (Table 7.3). The increase in blood BHBA production from butyrate by rumen epithelial cells increased with time in both groups of calves (Me0 and Me14) and is in agreement with studies by Giesecke et al. (1979) and Bush (1988). In our study

the effect was more pronounced for Me14 calves compared to Me0 calves and can be associated with the higher calf starter intake by Me14 calves.

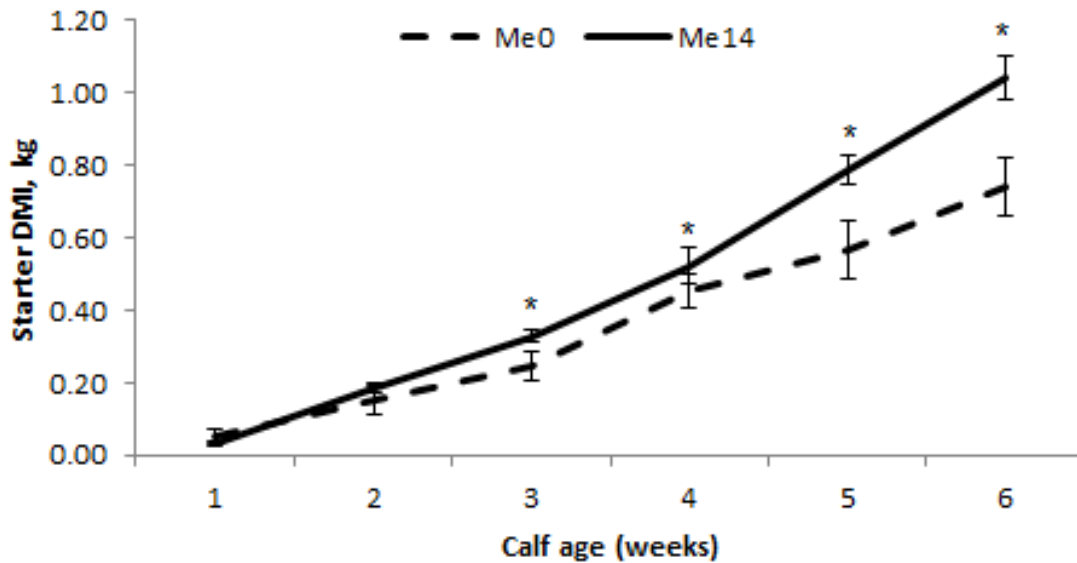


Figure 7.2. Starter dry matter intake for calves dosed (Me14) or not (Me0) with *M elsdenii*. *: Significant difference between groups ($P < 0.05$)

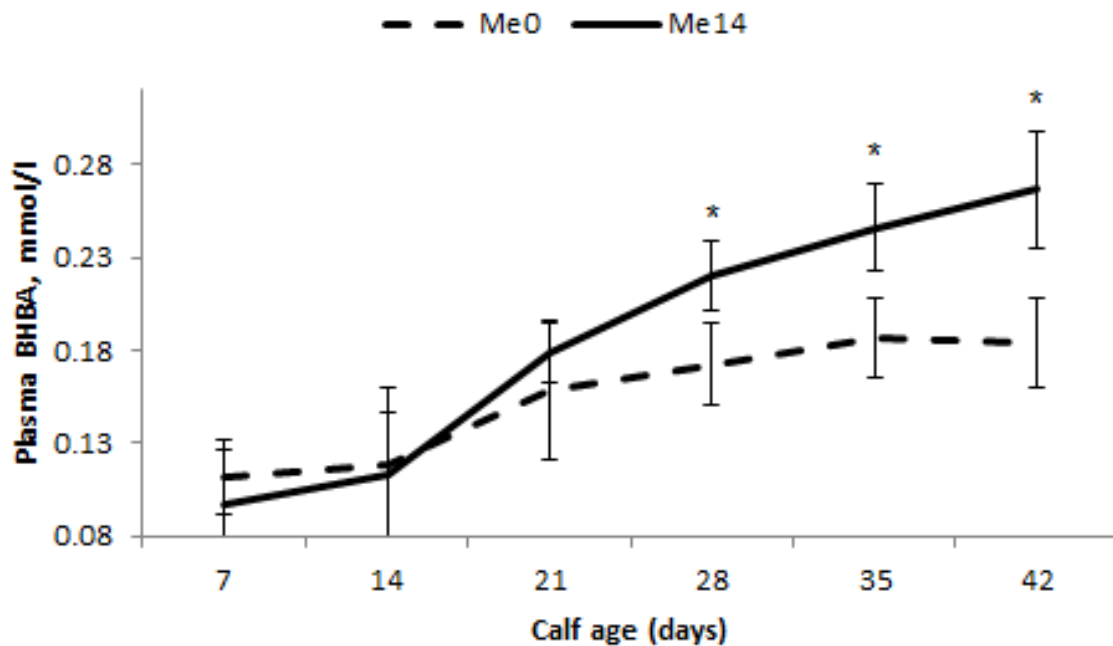


Figure 7.3. Plasma beta-hydroxybutyrate (BHBA) of calves dosed (Me14) or not (Me0) with *M elsdenii*. *: Significant difference between groups ($P < 0.05$)

Higher butyrate production could have increased ketogenesis in the rumen (Quigley et al., 1991) as more butyrate was available in the rumen for conversion to BHBA. Rumen butyrate

is oxidized by the rumen epithelial cells as it passes through the rumen wall (Quigley, 1991; Lesmeister and Heinrichs, 2004) and converted to BHBA.

7.3.2. Rumen parameters

Least square means of total and individual VFA concentrations are presented in Table 7.3. Total VFA (96.7 ± 5.6 mmol/l), acetate (53.7 ± 3.9 mmol/L) and propionate (24.2 ± 1.2 mmol/L) did not differ between treatments ($P > 0.10$), but butyrate concentration was higher in Me14 calves than Me0 calves ($P = 0.04$). Calves dosed with *M. elsdenii* NCIMB 41125 had higher reticulo-rumen weight ($P = 0.01$), papillae width ($P < 0.01$) and papillae density ($P = 0.02$) compared to Me0. No differences in rumen wall thickness (1.58 ± 0.1 cm) and papillae length (1.48 ± 0.1 cm) were observed between the two groups ($P > 0.10$).

Dosed calves had heavier reticulorumen weights with more muscle mass ($P = 0.010$) and more dense, and wider papillae ($P < 0.05$) when compared to control calves. Papillae length was not affected, although numerically higher for dosed calves compared to control calves. According to Leistmeister et al. (2004b), papillae length (PL), papillae width (PW) are first, and secondary in importance, respectively, as a rumen development variable. Leistmeister et al. (2004b) also found a positive correlation between PL and PW, suggesting that a high probability that a change in one parameter will reflect a similar change for another.

However, the magnitude of change seems to differ between the two parameters. In some studies, both PL and PW were increased at weaning for late weaned compared to early weaned calves (Zitnan et al., 2005) and for calves fed yeast culture (Lesmeister et al., 2004a). In another study, calves fed steam-flaked corn had greater PL when compared to calves fed dry rolled corn, but no change in PW was observed (Lesmeister et Heinrichs., 2004).

Table 7.3. Least square means for rumen volatile fatty acid concentration and ruminal development parameters taken at 42 days of age from calves dosed (Me14) or not (Me0) with *M. elsdenii*.

Items	Treatments		SEM	P
	Me0	Me14		
Rumen volatile fatty acid production				
Total VFA, mmol/L	96.2	97.2	8.681	0.882
Acetate	53.8	53.6	3.883	0.962
Butyrate	10.7	15.9	1.279	0.037
Propionate	24.3	24.1	1.175	0.915
Rumen development				
Reticulo-rumen, kg	1.05	1.17	0.0246	0.010
Rumen wall thickness, cm	1.60	1.56	0.102	0.826
Papillae length, cm	1.34	1.62	0.105	0.182
Papillae width, cm	0.86	1.17	0.027	<0.001
Papillae density, number/cm ²	87.1	97.6	2.343	0.023

Values in the same row differ if P < 0.05 and tend to differ if P < 0.10

Our results indicate that dosing *M. elsdenii* stimulated rumen butyrate and plasma BHBA production, and resulted in an accelerated rumen function development, suggesting more energy for thickening of the rumen wall, formation of papillae, and increasing capillary development, which is in agreement with results published by Weigand et al. (1975).

The association of rumen epithelial cell proliferation with increased plasma butyric acid was previously reported after dietary supplementation with sodium butyrate (Gorka et al., 2011) and also with infusions (Mentschel et al., 2001) of sodium salt of butyric acid to dairy calves. These reports suggested that the greatest effect on rumen papillae development was due to the high rates of mucosa cell apoptosis under the influence of butyric acid or high rates of butyric acid metabolism in mucosa cells. Reticulum weight is associated with increased BW (Tamate et al., 1962). Gorka et al. (2011) observed that dietary supplementation with butyric acid increased reticulorumen weight and papillae length and width in dairy calves fed limited amounts of milk (10% BW). Dosing *M. elsdenii* NCIMB 41125 resulted in higher calf starter intake, which could have additionally stimulated reticulorumen development (Tamate et al., 1962; Kristensen et al., 2007) as a result of consuming a greater volume of feed and resulting higher intake of energy and protein (Gorka et al., 2011) leading to better growth. Direct effects on cellular gene expression within the rumen (Glauber et al., 1991), and an increase in blood

flow through the rumen (Sander et al., 1959) are also suggested as mechanisms by which butyrate may stimulate papillae development.

However, mechanisms regulating rumen development and nutrient accessibility for developing calf tissues need further investigations. The biochemistry of plasma BHBA and rumen butyrate, feedback mechanisms with rumen epithelial metabolic activities and induction of papillary development are not well defined. More scientific clarity on these mechanisms would increase efficiencies in prediction of growth rates and management of dairy calves. The long-term effects of dosing *M. elsdenii* on rumen microbial populations and gut development and animal performance urgently need further research attention. Type of system, i.e. amount of starter intake significantly impacts on the effect of dosing on increasing not only starter intake but also feed efficiency.

7.4. Conclusion

Dosing calves on d 14 with *Megasphaera elsdenii* improved starter DMI, BW gain, and rumen development. Nevertheless, biochemical and physiological feedback pathways and the long-term survival of the strain of *M. elsdenii* in the rumen should be further investigated as well as long-term effects on performance, especially with regard to conventional and accelerated growth feeding systems.

CHAPTER 8

GENERAL DISCUSSION

8.1. Overview of studies

The first study was performed with the objective to investigate the survival of *M. elsdenii* NCIMB 41125 in the rumen, colon and rectum of young Holstein calves after an oral dosing, and evaluate effects on rumen fermentation and blood metabolites. In this study, it was found that *M. elsdenii* NCIMB 41125 colonizes both the rumen and colon, and that an increase in the proportion of butyric acid was observed when calves were dosed on both day 7 and 14 and then measured 72 hours later. Propionic acid was not affected by dosing. Therefore, we designed a second study to evaluate the effects of an oral dose and time of dosing of *Megasphaera elsdenii* NCIMB 41125 on intake, ADG, shoulder height, hip height, hip width, chest circumference, and incidence of diarrhoea in pre-weaned Holstein calves to determine the dosing time that would lead to better performance. Calves were fed restricted milk, typical of a conventional calf growth system. We observed an increase in starter feed intake when *Megasphaera elsdenii* NCIMB 41125 was dosed on day 7 and 14, and that the average number of days that diarrhoea was observed was decreased by 14 and 7% in Me7 and Me14 compared to the control, respectively. Although dosing at both d 7 and d 14 resulted in the same growth performance, dosing at d 14 was always numerically better, and was therefore considered as the best treatment. The third study was then performed with the objective to compare the effects of *M. elsdenii* NCIMB 41125 on intake, growth and blood BHBA during the pre-weaning period and two weeks after weaning. Calves were fed milk *ad lib* at feeding time, therefore representing an accelerated growth feeding system. We observed that administering *M. elsdenii* NCIMB 41125 to Holstein calves at 14 d of age improved pre- and post-weaning performance and starter feed intake compared to control calves. Improvements in starter feed intake suggest greater ruminal VFA concentrations to stimulate rumen development. In addition higher blood beta-hydroxybutyrate concentrations observed in dosed calves may indicate greater metabolic activity of the rumen epithelium. Based on this results, it was decided to perform a fourth study to evaluate rumen development and fermentation as affected by the administration of *Megasphaera elsdenii* NCIMB 41125. The oral dose of *M. elsdenii* NCIMB 41125 improved starter DMI and tended to improve ADG and weaning BW. Calves dosed with *M. elsdenii* NCIMB 41125 had greater reticulo-rumen weight, papillae width and papillae density than control calves. No differences in rumen wall thickness and papillae length were observed

between the two groups. Total VFA's, acetate and propionate did not differ between treatments, but butyrate concentration was higher in dosed calves than control calves.

Based on the abovementioned results, some of the most important outcomes of the research will be discussed in more detail in the following section.

8.2. Performance

In all four studies, dosing *M. elsdenii* NCIMB 41125 to calves consistently increased starter DMI during the pre-weaning period. Improved starter DMI was observed both when feeding limited amount (study 2 and 4) and high levels of milk (study 3). When comparing the starter DMI of calves dosed with *M. elsdenii* NCIMB 41125 at 3 different days after calving (age), dosing at d 14 resulted in a significantly higher starter DMI than dosing at d 21, but only numerically higher than when dosing at d 7. Milk intake was lower for dosed calves having free access to milk as a result of the high starter intake compared to calves fed according to a conventional growth system. Earlier studies have reported the rumen fermentation characteristic, metabolism of VFA's and amino acids, and their role in ruminal metabolism (Counote et al., 1981; Wallace, 1986; Marounek and Barlos, 1987). *Megasphaera elsdenii* has been administrated in adult ruminants for its ability to control lactic acid accumulation in the rumen (Meissner et al., 2010), and variable responses were observed. Increased feed intake was reported in lambs (Henning et al., 2009), steers (Henning et al., 2010) and cattle (Drouillard, 2004) after drenching with *M. elsdenii* NCIMB 41125. In contrast, no effect on feed intake was observed by Leeuw et al. (2009) in a study with feedlot steers.

The dynamics in the developing GIT of young calves as well as the micro-organism's population is totally different compared to the adult ruminant. Increasing the rate of DMI is the more important parameter in early weaning management systems, as it can impact on growth after weaning (Greenwood et al., 1997). In newborn calves, solid feed intake depends on the development of rumen capacity and rumen wall development (Khan et al., 2007; Kristensen et al., 2007) as well as rumen microflora and papillae, which affect the efficiency of nutrient digestion and absorption. Because it was assumed and later demonstrated in the present study that administration of *M. elsdenii* NCIMB 41125 could directly stimulate the surface available for absorption of fermentation end products through stimulation of rumen epithelium development, it may explain the higher starter DMI of dosed calves.

In conventional feeding systems (restricted milk feeding), the oral dosing of *M. elsdenii* NCIMB 41125 did not affect calf ADG in one study (study2), but tended to improve calf ADG in another (study 4), and the significant increase in calf starter in both studies did not translate into a higher gain:feed ratio. However, when calves had free access to milk during feeding time, dosing *M. elsdenii* NCIMB 41125 improved ADG during both pre- and early post-weaning periods. In addition and in contrast to the studies on a conventional system, the increased starter intake, although at lower level, resulted in increased feed efficiency for Me14 calves. The ADG has always been reported to be higher in calves fed higher volumes of milk compared to calves fed lower milk (Brown et al., 2005; Bartlett et al., 2006), but the increasing effect of *M. elsdenii* NCIMB 41125 on ADG observed only in calves having free access to milk during feeding time is unclear. However, it should be noted that when calves had free access to milk, metabolisable energy from milk constituted a lower portion of the total metabolisable energy intake for dosed calves compared to control calves. Therefore decreasing milk intake should not impact these calves as much as the control calves, because they are already relying more on the starter feed as a source of energy. This supports the concept that calves dosed with *M. elsdenii* NCIMB 41125 can be weaned at an earlier age.

Dosing *M. elsdenii* NCIMB 41125 improved the gain:feed ratio of calves having free access to milk (study 3), but did not affect it when calves received restricted milk (study 2 and 4). The amount of milk fed to dairy calves influences starter feed intake and growth (Appleby et al., 2001). The restricted milk feeding in a conventional system is generally associated with depressed performance due to low nutrient availability (Appleby et al., 2001), while greater milk consumption in accelerated growth systems improves growth (Jasper and Weary, 2002), but delay intake of starter (Appleby et al., 2001; Hammon et al., 2002). Increasing body gain per unit of nutrient intake become important as growth is obtained for the same amount of maintenance cost. In this regards, the results suggest a benefit of dosing *M. elsdenii* NCIMB 41125 for calf on accelerated growth and perhaps in early weaning because of increased starter intake. In early weaning systems, emphasis is more on increasing starter intake.

As mentioned above, one of the major disadvantages of raising calves on accelerated-growth feeding programs, is a reduced starter intake. Thus, it probably decreases the amount and quality type of rumen metabolites, which may delay VFA's absorption by rumen mucosa. Consequently, the liver shift from glycolytic to gluconeogenic metabolism might also be delayed. Rumen mucosa development can also be affected when feeding high amounts of milk,

especially papillae growth. From our study it can be concluded that an oral dose of *M. elsdenii* NCIMB 41125 administered to calves on an accelerated- growth feeding program had great potential to stimulate rumen mucosa development.

Dosing *M. elsdenii* NCIMB 41125 improved the weaning BW of calves when fed both high and restricted amounts of milk, which may also be attributed to increased nutrient availability due to early increased starter DMI, and consequently improved ruminal micro-organism and fermentation activities (Anderson et al., 1987). Improved DMI observed in these studies are in agreement with Miller et al. (2013). In the latter study, DMI and ADG during the 64 days' study period was greater in high risk calves dosed with *M. elsdenii* at processing compared to high risk control calves during the receiving period. In addition, morbidity and Bovine Respiratory Diseases therapeutic treatment were significantly decreased.

8.3. Blood BHBA, rumen fermentation, and rumen development

In the study where calves were having free access to milk, oral dosing with *M. elsdenii* NCIMB 41125 increased plasma BHBA concentrations from the week after dosing. Higher plasma BHBA levels were maintained until weaning at 56 days. In the study where calves received a limited amount of milk, plasma BHBA concentration was increased when calves were dosed with *M. elsdenii* NCIMB 41125. Plasma BHBA is widely recognized as an indicator of rumen development in new born calves (Quigley et al., 1991), its greater concentration in calves dosed with *M. elsdenii* NCIMB 41125 can therefore be associated with earlier rumen function and rumen development.

In the final study, where rumen fermentation and papillae development were evaluated in euthanised calves, oral dose of *M. elsdenii* NCIMB 41125 resulted in higher reticulo-rumen weight, papillae width and papillae density, with no effect on rumen wall thickness and papillae length. Total VFA's, acetate and propionate were also not affected, but butyrate concentration was increased with *M. elsdenii* NCIMB 41125. These positive effects of dosing with *M. elsdenii* NCIMB 41125 on rumen development, together with increased ruminal molar proportion of butyrate are associated with higher plasma BHBA observed in the same groups of calves. This indicates a greater metabolic activity of the rumen epithelium due to increased molar proportion of butyric acid in the rumen and improved starter intake as a result of dosing with *M. elsdenii* NCIMB 41125. The role of butyrate, as energy source during rumen epithelial

cell's growth, over glucose has been discussed earlier (Giesecke et al., 1979). Glucose is the main source of energy for calves prior to ruminal development, but as consumption of solid feeds increases and the rumen begins to develop, the contribution of VFA's to the calf energy requirements also increases, resulting in elevated concentrations of BHBA in plasma (Quigley et al., 1991).

Most of the ruminant animal's energy requirements are met by the VFA's absorbed through the epithelial lining of the rumen (Annison and Armstrong 1969) with less than 10% of the VFA's passing into the small intestine (Goosen 1976). The primary ruminal VFA's (acetate, propionate and butyrate) are metabolized to different extents by the ruminal epithelium. These metabolites are then converted into ketoacids and provide energy during synthesis of other substrates (Beharka et al., 1998). The ruminal epithelium is the primary source of circulating ketone bodies (Heitmann et al. 1987) and oxidizes butyrate and converts it to BHBA through the rumen wall (Lesmeister and Heinrichs, 2004). Approximately 90% of ruminally absorbed butyrate carbon appearing in portal blood is in the form of BHBA and acetoacetate (Beck et al. 1984).

The ketogenic capacity of the rumen epithelium to produce D-3-hydroxybutyrate from butyrate increases with age independently of solid feed intake (Lane et al., 2000), while the ability of the rumen mucosa to absorb volatile fatty acids increases with solid feed intake, but not with age (Sutton et al., 1963). However, variation in *M. elsdenii* and *M. elsdenii* NCIMB 41125 populations should be investigated as well as the long term effects on post-weaning growth and also lactation performance. Lactation performance is influenced by early development. Therefore improved calf growth rate before weaning is a major environmental factors influencing the expression of the genetic capacity of the animal for milk yield (Soberon et al., 2012), and can be manipulate via early calf nutrition. Interactions with other volatile acids in relation to the feed composition as well as the effect of alterations in ruminal pH and total VFA's concentration also needs to be studied in more detail using both *in vivo* and *in vitro* studies to refine our understanding of the role of ruminal epithelial metabolism on the energy metabolism of the whole animal and the mode of action of *M. elsdenii*.

CHAPTER 9

CONCLUSION

Overall, results from all the studies suggest a benefit on general calf performance when administrating *M. elsdenii* NCIMB 41125 to calves fed under both conventional and accelerated growth systems during the pre-weaning period.

Based on the results obtained from this series of studies, the following conclusions can be made:

1. *Megasphaera elsdenii* NCIMB 41125 can colonize, establish and survive in the gastrointestinal tract of new born calves after being administrated orally.
2. Administrating *M. elsdenii* NCIMB 41125 to new born calves at d 14 of age has potential to improve starter DMI in calves following an accelerated growth as well as a conventional milk feeding program.
3. The effects of *M. elsdenii* NCIMB 41125 on improving feed efficiency was more beneficial in accelerated-growth and early weaning calf rearing system.
4. Administrating *M. elsdenii* NCIMB 41125 to new born calves at d 14 in an accelerated-growth feeding program improved solid feed intake during the pre- and early post-weaning periods, and improved BW and ADG after weaning.
5. Administrating *M. elsdenii* NCIMB 41125 to new born calves at d 14 improved rumen fermentation and development in terms of reticulo-rumen weight, papillae length, papillae width, papillae density and rumen wall thickness

However, mechanisms regulating papillae development and differentiation in relation with nutrient accessibility, changes between plasma BHBA and rumen VFA's and their relationships require more understanding. Understanding these mechanisms will enhance nutritional programs for more precise calf rearing. In addition, more research with different qualities of milk replacer and long-term effects of dosing *M. elsdenii* on GIT development and performance should also be conducted.

CHAPTER 10

CRITICAL EVALUATION

The objective of the first study was to evaluate the survival of *M. elsdenii* NCIMB 41125 in the GIT after dosing at different ages. Despite all the measures taken to avoid cross contamination, the strain was found in calves not dosed, suggesting that contamination occurred, probably that calves were able to touch one another. It could be also that a feeding bucket may have been allocated wrongly or were not cleaned properly before being wrongly allocated. This also could have happened in the other trials and could have influenced measured parameters, therefore impacting on the results of the trial. Ensuring hand washing before handling successive calves, proper cleaning of equipment after every measurement or using different equipment between treatment groups as well as proper identification of feeding and measuring equipment can help avoid or minimising contamination.

Another factor to consider is the small size of the herd used to supply calves for these experiments. The duration of the experiment was therefore extended in order to have enough calves to complete the study. This resulted in calves entering the trials in different seasons, which can affect parameters such as body weight, heat for example impacts on growth. It was reported that calves born in the summer and raised in outdoor hutches on whole milk gained significantly less weight compared to calves born in fall in a temperate climate (Broucek et al., 2007). Calves reared in a hot temperature of 27°C were reported to have gained 8.6 kg less in three months than calves raised under a cooler temperature of 10°C (West, 2003). Recent research correlated calves born in winter with a greater risk of developing diarrhea (Gulliksen et al., 2009). If a small herd size is a given, a proper blocking procedure should be followed to have equal numbers of summer/winter calves. The ideal situation would be to purchase a large batch of calves just after calving from the same supplier.

In the experiment evaluating the effect of *M. elsdenii* NCIMB 41125 on rumen development, we investigated only BHBA and volatile fatty acids. Papillae development is a mechanism that still requires more understanding, and additional parameters should be investigated. Beside butyric acid, propionic acid is believed to play a secondary role on rumen epithelium cell development and proliferation, and could be a supportive measurement when evaluating rumen development. Propionic acid is the VFA metabolized in the second largest quantity during absorption, after butyric acid, and contributes to rumen mucosa development. In addition, the

possibility that butyrate and propionate metabolism by the ruminal epithelium induces an increase in blood flow through the rumen and (Sander et al., 1959), as well as their possible direct effect on gene expression within the rumen (Glauber et al., 1991) are amongst the mechanism by which the two VFA's stimulate rumen development.

Health parameters were measured in one of the studies to evaluate the probiotic potential of administrating *M. elsdenii* NCIMB 41125 to pre-weaned calves, but no stress conditions or other harsh condition were created in order to allow for the bacteria to express its potential, perhaps to a larger extent.

Colonization implies adherence and multiplication on mucosal epithelium (Ewaschuk et al., 2004). It is possible that the *M. elsdenii* NCIMB 41125 may have been growing in intestinal contents, but not adhering to mucosal cells. Further investigation of mucosal adherence in calves is required to more clearly determine if mucosal colonization occurs. In addition, our knowledge about the changes in *M. elsdenii* and *M. elsdenii* NCIMB 41125 populations over time especially after weaning should be investigated further. This would require repeated rumen sampling. On the other hand, reliable methods of ruminal fluid sampling are lacking. Obtaining rumen fluid from young calves is associated with practical problems, as it may disturb solid feed intake for several days.

The significance of impact of dosing with *M. elsdenii* NCIMB 41125 in different calf feeding systems vary. Results suggest better performance in term of efficiency in accelerated growth than conventional feeding systems. A range of studies should be performed from an early weaning system to a number of studies where increasingly high volumes of milk are fed up to *ad lib* during the full day. This would indicate under which conditions the role of *M. elsdenii* can be optimized. The use of appropriate photographic and video equipment should be considered for a visual evaluation of the change in rumen development in support of physical papillae measurements.

CHAPTER 11

LITTERATURE CITED

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