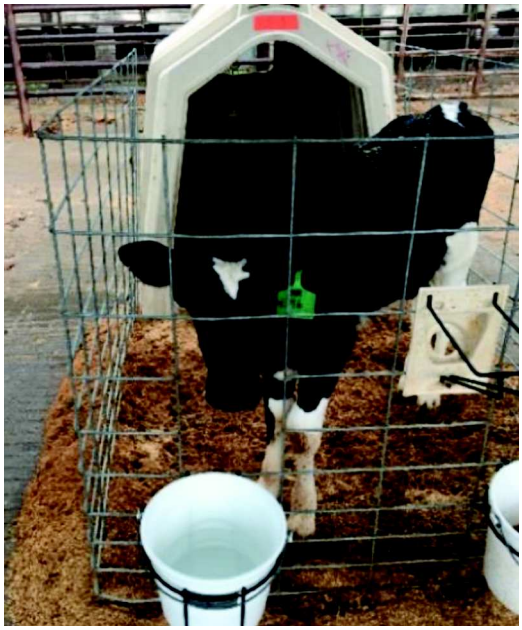
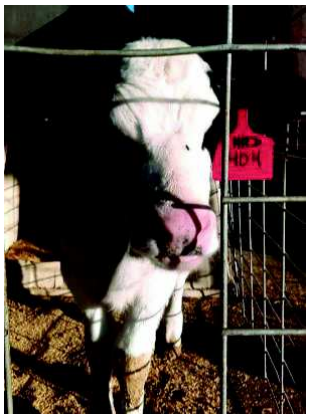
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

# The effect of dietary supplementation of monobutyrin on growth, health, intestinal and rumen morphophysiology of preweaning Holstein bull calves





## Master's Thesis

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# Preface

This project was conducted from October 2016 to June 2018 at the Faculty of Health and Medical Science, University of Copenhagen, Frederiksberg as a part of the master education in Animal Science. The experimental part was carried out at University of California, Davis, Department of Animal Science from October 2016 to December 2016, and laboratory analysis from January 2017 to June 2017. The purpose of this project was to research the effect of monobutyrin added in MR for preweaning calves on the rumen and small intestinal development.

I would like to thank my main supervisor Mette Olaf Nielsen at the Department of Veterinary and Animal Sciences, University of Copenhagen, for her guidance throughout writing the thesis. Also, a big thank you to my co-supervisor Peng Ji from the nutrition department at University of California Davis, for putting me on this project and guidance throughout the experimental part of my thesis. Thank you to Richard Sygall from Perstorp Feed and Food, Malmö, Sweden, whom this study was carried out in co-operation with.

The research project was carried out at the UC Davis beef cattle facilities, a big thank you to Don Harper, Animal resource manager at the beef facility and to Joslyn Mendez being responsible for the interns and calves and in co-operation with Joanna Martinez doing the RNA extraction. Thank you to all the interns on the project for the helping with the calves, gathering data. Thank you to Dr. Helen Raybould and Amy M. Erlich for carrying out the permeability experimental part. Furthermore, a thank you to Simone Sohrbeck Thorsager Billing for helping me through this writing process. I also owe Rajan Dhakal, research assistant at Department of Veterinary and Animal Sciences a thank you for helping me with my statistics, correcting my coding, helping write the code for health data and discussing my results with.

I owe my deepest gratitude to Lemvigegnens Landboforening especially the dairy consultants department, for giving me an office to write my thesis, all their support, help, humor and happy presence for the past 10 months.

At last, thank you to my family and friends for all the support and patience the past 2 years during the experimental trial, laboratory work and thesis writing.

# Abstract

The newborn calf is born as a monogastric animal. The abomasum is developed but the forestomachs are yet undeveloped. The development of the rumen and small intestine is affected by the form of the feed and the nutrients in the feed. The young calves' gastrointestinal tract (GIT) is adapted to utilize nutrients in milk, and as the calf get older and eat more solid feed the GIT develops and adapt. Rumen and small intestine is developing, in the young calf, this development is affected by nutrients. Medium and short chain fatty acids (SFCA) can increase growth and promote rumen development. Especially the SCFA butyrate has a stimulatory effect on rumen papillae development. In this, it was hypothesized that SCFAs, more specific butyrate, plays an important role in calf growth, calf health, rumen development and small intestinal development of the preweaning and weaning calf

From the literature review, it is clear that the best source of carbohydrates, protein and fatty acids for the calf is cow milk or milk preruminant replacer (MR). Especially SCFAs like butyrate, which is naturally present in cow milk, seem to have a positive effect on calf growth, calf health, rumen development and intestinal development in preweaning and weaning calves. From these studies, it was concluded that feeding 3 g/day of Na-butyrate two times a day promotes calf growth and rumen development and has a tendency to prevent scours. Thus, the aim of the experimental study was to investigate what affect monobutyrin could have on growth performance, feed intake, health factors, rumen papillae and jejunum epithelial development in preweaning dairy calves. Calves were brought in at an age of ≤ 4 days and euthanized at an age of ~56 days. Daily measurements of feed intake and water intake were kept, weekly recordings of body weight and biweekly body frame measurements were kept.

According to results found in the literature and in the present study, it was concluded that supplementing butyrate in MR seemed to have a positive effect on calf growth, calves were generally healthy throughout the study. Rumen development was positively affected by supplementation of Na-butyrate but monobutyrin seemed to have a negative effect on papillae length and papillae length to width ratio. Results on intestinal development were contradictory but it was concluded that the relative mRNA expression of tight junction proteins was positively affected by monobutyrin supplementation. Absorptive capacity may be improved and thereby possibly increasing intestinal integrity.

# Table of contents

# 1. Background

Calves are born as monogastric animals because the rumen, reticulum and omasum are not fully developed, this means that fermentation chambers are not yet fully developed to the extent where fermentation of feed can take place. Milk ingested by the young calf bypasses the rumen due to an esophageal groove reflex which induces muscle flaps to curl and form a tube leading the ingested milk from the esophagus through the forestomachs. This reflex is stimulated by milk and by suckling the teat or bottle (Comline and Titchen, 1951; Guilloteau et al., 2010). When calves start to eat solid feed, like starter grain, the metabolic and physical development of the rumen begins. Increased rumen weight and muscular development are stimulated by the physical form of the feed, particularly coarse feed like roughage. However, physical form does not have an effect on papillae development. It has been demonstrated that certain short chained fatty acids (SCFA), present in milk fat and produced during rumen fermentation play a special role in stimulating ruminal fermentation and development of papillae in the rumen (Baldwin et al., 2003; Hamada et al., 1976; Žitňan et al., 1999).

The most potent of these appears to be butyric acid which is a SCFA naturally present in triglycerides in dairy milk (Månsson, 2008) but it is also one of the SCFA produced in the rumen during fermentation (Guilloteau et al., 2010). Dietary supply of butyrate, in particular sodium butyrate (Na-butyrate), has been shown to have a positive effect on calves' health. It has been seen that supplementing Na-butyrate gives less thin feces and calves with diarrhea receiving Na-butyrate supplement required less days on electrolyte treatment compared to a control group (Gorka et al., 2009; Górka et al., 2011a). Guilloteau *et al.* (2009) also found that Na-butyrate could stimulate cytoprotection in the intestines of calves. Thereby, supplementing butyrate in milk replacer (MR) to calves could potentially improve their health.

Feeding sodium butyrate to weaning piglets has been shown to promote growth performance and development of the intestinal mucosa (Kotunia et al., 2004; Le Gall et al., 2009; Tonel et al., 2010). However, it has been seen in cell cultures with the human colonic epithelial cell line Caco-2 cells that a high dose (8 mM) of butyrin compared to a low dose (2 mM) could disrupt the mucosal barrier function and with the high dose there was an increased rate of apoptosis in the intestinal epithelial cells (Peng et al., 2007).

Supplementing sodium butyrate to calves has been shown to enhance growth performance and rumen development, possibly due to stimulation of epithelial cell proliferation, differentiation and decreasing apoptosis (Górka et al., 2011a; Guilloteau et al., 2009b; Kato et al., 2011). Supplementation of Na-butyrate also appears to have a positive effect on the health of

calves, possibly through cytoprotection (Górka et al., 2011a; Guilloteau et al., 2009b). Supplementing Na-butyrate to 44 Holstein male calves at the age of 12 days has been shown to increase pancreatic elastase II activity and dipeptidase IV in duodenum. Elastase II could enhance digestibility of proteins while dipeptidylpeptidase IV increases activity of maltase and lactase, where lactase breaks down lactose in the milk and maltase breaks down maltose (Guilloteau et al., 2009b). When supplementing Na-butyrate in vitro to a culture of cdx2-IEC cell monolayer, which was used to mimic the barrier function, an increase in tight junction proteins has been seen in the barrier function, which could mean an increase in paracellular permeability in the barrier function. However, effect of butyrate on tight junction proteins has not yet been studied extensively in vivo (Wang et al., 2012). In cattle, especially dairy cattle, increased development of the rumen and intestines in the weaning calf could cause an increase in surface area of the rumen and intestines and possible an increase in digestive enzymes like elastase II and dipeptidylpeptidase. If this effect is permanent and persists in the adult cow, and not just seen when the calf is developing the gastro intestinal tract (GIT), this could result in a better nutrient uptake and thereby a higher milk yield potential.

## 1.2 Research questions and hypothesis

In this study, it was hypothesized that SCFAs, more specific butyrate, play an important role in GIT development, calf growth and calf health of the preweaning and weaning calf.

To address this hypothesis, a literature review was carried out to identify factors that could influence rumen and small intestinal development, with special emphasis on the impact of butyrate in different forms. Furthermore, an experimental study was conducted in preweaning calves to determine:

- whether feeding MR with different doses of monobutyrin would enhance preweaning calves' growth.
- If stimulation of the preweaning calves' development of rumen and jejunum epithelia had positive implications for performance and health
- And to establish what dose would be optimal.

The study was delimited to look at calves up to 8 weeks of age, at which age weaning started. The study was focused mainly on the development of rumen and jejunum, since these are major sites of absorption in the calf/cow.

The literature review addresses the following questions in order to look at rumen and small intestinal development, and influence of nutrient intake on rumen and intestinal development:

- How does the newborn calves GIT look like and function?

- How does the newborn calves GIT develop over time from monogastric to functional ruminant?
- What source of nutrients is the preweaning calf's GIT designed for?
- What effect does protein, carbohydrates and fat have in the developing GIT in calves?
- What are the results on research in butyrates effect on the calves developing GIT?
- What effect will the supplementation of butyrate in the preweaning calf have long-term on the cow?

**Research:**

The literature review raised more questions on what affect monobutyrin could have on growth performance, feed intake and health factors such as diarrhea and respiratory health of preweaning dairy calves. Furthermore, in relation to these parameters, it would be interesting to investigate rumen papillae and jejunum villus development in preweaning dairy calves. Following questions were investigated:

- Can supplementation of butyrate to the calf increase growth, measured as Body weight and body frame (wither height, hip height, hip width, heart girth and body length)?
- Can supplementation of butyrate to the calf improve health?
- Can supplementation of butyrate to the calf increase rumen papillae length and width, intestinal villus length and crypt depth?
- Which dose would be optimal, comparing a high (0.75% of DM) to a low (0.37% of DM) dose?
- Furthermore, in relation to these parameters, it would be interesting to investigate rumen papillae and jejunum villus development in preweaning dairy calves.

Based on these questions, a research trial was conducted.

# 2. Introduction

The aim of this chapter is to identify specific nutrients that could be important in stimulating GIT development. To understand why these nutrients could be important, basic understanding of the preweaned calves GIT is needed. To identify which nutrients and end products from digestion of these nutrients that could have an effect on GIT development, the degradation and absorption of protein, carbohydrates and fat is in focus. Focus will be on the rumen and small intestine. This chapter will review the development of the calves' GIT going from monogastric to ruminant. End products from rumen fermentation will also be drawn in to see if SCFAs could have a positive effect on GIT development. Furthermore, the SCFA: acetate, propionate and butyrate will be compared to see their effect on the development of the GIT, with special emphasis on butyrate.

## 2.1 The calves' digestive tract

Newborn calves are not born with a competent immune system. Structure of the ruminant placenta prevents prepartum transfer of immunoglobulins (Ig), immune cells and various cytokines (Barrington and Parish, 2001). The first milk the cow produces after calving is colostrum that contains immunoglobulins (Ig), immune cells and various cytokines, which the calf needs in order to be protected against pathogenic microorganisms, various viruses and bacteria. Colostrum is important because this is how the cow transfers immunity to the calf, not through the placenta. (Gapper et al., 2007; Uruakpa et al., 2002). These Ig's, immune cells and cytokines are absorbed by passive transfer (Blum and Hammon, 2000; Hurley and Theil, 2011). Within 6 hours after birth, the absorption rate of Ig's decline rapidly, and after 24 hours only few calves will still be able to absorb small amounts of Ig. It is important that the intestine closes for the absorption of macromolecules, since there is a high risk of absorbing viruses and bacteria with the open intestine (Matte et al., 1982; Morin et al., 1997; Stott et al., 1979). Colostrum contains colostral trypsin inhibitor which inhibits trypsin and chymotrypsin, thereby antibodies, which are proteins, in colostrum can avoid proteolysis and be absorbed by epithelial cells in the small intestine through pinocytosis. This helps the calf to be able to absorb the colostral proteins that constitute the passive immune defense of the calf (Sehested et al., 2003; Sjaastad et al., 2010).

When the calf is born, it is born as a monogastric animal. The abomasum is developed, whereas forestomaches are as yet undeveloped. In the newborn calf that only drinks

milk and does not eat any grain or forage, the so-called esophageal groove reflex prevents milk from going into the forestomachs. The esophageal groove consists of muscle flaps extending the esophagus, and when the calf is suckling the reticular groove contracts reflexively. When the reticular groove contracts the muscle flaps shortens and curl, forming a tube, which leads the ingested milk straight into the abomasum, hence bypassing the forestomachs (Sjaastad et al., 2010). In figure 2.1, the preruminant calves' digestive tract is shown, compared to the ruminant cows' digestive tract, as seen on the picture, the forestomachs are yet undeveloped in the preruminant.

The GIT has three major phases of development in the calf fetus. At first mucosal cells undergo proliferation and morphogenesis, then cells differentiate - different and distinctive cell types appear, lastly, both as a fetus and as a neonate the GIT has a period of maturation. In this maturation period, the intestines develop into being able to transport luminal contents, digesting and absorbing nutrients. These three phases are not sharply delimited, but a substantial part of the last phase, the third phase, occurs postnatally. The digestive secretion and absorption of nutrients are important factors in regulating the volume and composition of body fluids, and the secretion and absorption is controlled by hormones and peptides. Regulating the volume and composition of body fluids is especially critical during the transition from fetus a neonate and again, when the calf goes from milk feeding to solid feed and becomes a functional ruminant (Guilloteau et al., 2002). Therefore, it is important that the calf gets the right nutrients, nutrients available for the calf to digest as a preruminant, in order to secrete and absorb the nutrients needed to regulate the volume and composition of body fluids.

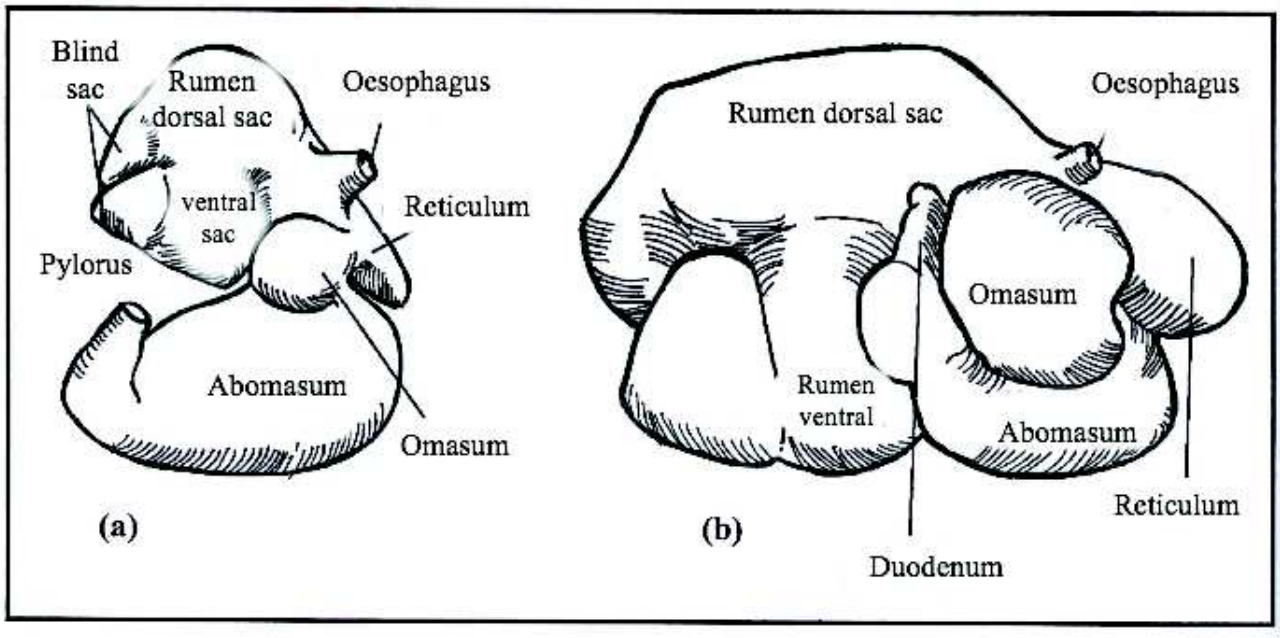



**Figure 2.1 digestive tract of (a) The preruminant calf and (b) the ruminant cow**

## 2.2 Development of the rumen

In the preruminant calf, the rumen is still undeveloped. As the calf gradually becomes a ruminant, the rumen develops and papillae appear in the wall of the rumen. Papillae has no smooth muscle and are thereby immobile, the purpose of papillae is to increase the surface of the rumen and the capacity for absorbing nutrients (Sjaastad et al., 2010).

The physical form of the diet has an effect on development of the rumen. The structure in the roughages contributes to an increased weight of the rumen by stimulating muscular development, and expanding the rumen, leading to a bigger rumen volume. (Baldwin et al., 2003; Meale et al., 2017; Mirzaei et al., 2015). Roughages also stimulate saliva flow to the rumen. All of these factors influenced by roughages also contributes to stimulation of rumination in the calf (Meale et al., 2017). Chopped forage like alfalfa, increases ruminal pH, which gives an environment where microbial bacteria can live (Beharka et al., 1998; Castells et al., 2013; Mirzaei et al., 2015). Concentrate feeds are also needed for development of papillae and for fermentation in the rumen to happen. Production and absorption of rumen fermentation end products, which are SCFA's, are the main stimulants of rumen papillae development. This means that development of rumen papillae is limited, while the calf is only getting milk that does not enter the rumen to any significant extent, and thereby very little fermentation happens in the rumen of the neonatal calf (Baldwin et al., 2003; Meale et al., 2017; Zitnan et al., 1999).

## 2.3 Development of the small intestines

Due to the undeveloped forestomachs, almost all nutrient degradation and absorption in the newborn calf happens in the small intestine. The small intestine can be divided into: duodenum, jejunum and ileum. The small intestine has folds with villi that increase the absorption area of the intestine. When the calf is born, synthesis of some regulatory peptides and hormones is already occurring in the small intestine: Gastrin, CCK, Secretin, VIP, Motilin, PP, Somatostatin and GIP. It has been shown that a few days after birth and again around weaning the small intestine grows disproportionally faster than other organs in the body. The growth of the intestinal mucosa during the first 24 hours post-partum, is a result of a combination of colostral protein accretion and increased cell proliferation in the intestinal tissue (Guilloteau et al., 2002).

The growth of the small intestine could be affected by peptides and hormones. It has been seen that after the first colostrum feeding, there is an increase in plasma concentration of some of the gut regulatory peptides and hormones. For example, there is an increase in plasma

concentration of gastrin and CCK, this was associated with marked hyperplasia of pancreas in lambs, indicating that gastro intestinal growth was promoted (Guilloteau et al., 2002).

The microbial flora also seems to have an effect on GIT function in the young calf. The microbial flora starts colonizing in the intestines at birth. The whole effect on the development of GIT function is still not clear. The exposure to microbial flora increases DNA synthesis in the intestinal mucosa of ileum, as well as the distal and proximal jejunum. The microbial flora reduces the number of villous cells but increases the number of crypt cells in the distal part of the small intestine (Guilloteau et al., 2002). These findings suggest that microbial flora colonizing in the small intestines, participates in the development in the small intestine through regulation of endocrine cells and the epithelial structure of the small intestine.

## 2.4 Nutrient absorption and effect on gastrointestinal development

From the preceding section, it seems clear that the development of the forestomachs and small intestine are related to the ingestion of colostrum, milk and forage. Therefore, the next sections will review the specific effects that dietary protein, carbohydrates, starch, NDF and the short chain fatty acids formed during fermentation may have on the development of the GIT. This will include also a general description of how degradation of dietary products occurs in the young calf, since the young calf's digestive tract is adjusted to the digestion of milk component with a high digestibility.

### 2.4.1 Protein

In the newborn calf, degradation of protein starts in the abomasum and primarily occurs in the small intestine thereafter. Some of the cells that secrete enzymes, mucous and/or hydrochloric acid from the abomasum are called chief cells, partiel cells and mucous cells and together they are organized in so-called fundic glands. These cells produce chymosin, prochymosin and pepsinogen, all chief cells and some mucous cells produce prochymosin and pepsinogen. In the newborn and 1-day old calf, compared to calves 3 days old and 4 weeks old, the three types of cells have not developed fully yet. This means that the first day and up to 3 days after birth, the abomasum has a low secretion of pepsin but chymosin secretion is high. Because of the not fully developed fundic glands, the production of hydrochloric acid is also low the first days. This is how the IgG from colostrum is able to reach the small intestine intact (Andrén et al., 1982; Guilloteau et al., 1984; Tamate et al., 1963). The secretion of chymosin decreases after 1.5 days of age, and is basically not secreted in the adult cow. Whereas Secretion of pepsin is higher at calving in the calf than 3 weeks prepartum. Pepsin secretion increases

rapidly from around 71 days postnatally. In calves over 6 months, basically all chief cells and most mucous cells produce pepsin. This means in the foetus and the newborn calf, chymosin/pepsin ratio is high, it remains high the first 2 days after calving and then decreases significantly (Andrén et al., 1982; Guilloteau et al., 1984). The secretion of chymosin in the abomasum in relation to protein utilization and degradation is important in order for nutrients to be released to the small intestine adjusted to the enzymatic capacity in the small intestine. Chymosin binds much of the casein and fat in milk in the abomasum forming curd, within 10 minutes of feeding. From the abomasum, the nutrients are then slowly released to the small intestine over a period of approximately 24 hours (Longenbach and Heinrichs, 1998; Miyazaki et al., 2009; Yvon et al., 1984).

The pancreas secretes proteases and other enzymes (see later) to the small intestine, which digest dietary proteins and other nutrients. Right after calving and up until 1.5 days of age, the activity of the enzymes; chymotrypsin, trypsin, lipase, colipase and amylase is minimal. After 1.5 days of age, the activity increases rapidly (Guilloteau et al., 1984). Most of the milk proteins will be broken into peptides in the abomasum. For proteins to be absorbed in the small intestine, these peptides need to be cleaved into absorbable di-, tripeptides and free amino acids by pancreas proteases like trypsin, chymotrypsin, elastase and carboxypeptidase. If the peptides are not already broken down into free amino acids before absorption, the final breakdown of dipeptides and tripeptides into free amino acids happens during absorption, where they are rapidly hydrolyzed intracellularly by di- and tripeptidases to free amino acids (Caspary, 1992; Daniel, 2004; Leibach and Ganapathy, 1996). Hydrolysis of proteins is not the rate-limiting step for removal of proteins from the intestinal lumen, it is rather the transport of degraded products into epithelial cells (Sjaastad et al., 2010). The major part of these peptides is absorbed in the upper jejunum; however, this is not the only region for absorbing peptides. Presumably when the peptides pass through the small intestine, the proximal part will absorb more long peptides and as the proteins pass through the small intestine, proteases will cleave more peptides into amino acids and the distal part of the small intestine will absorb more amino acids. The absorption of protein will depend on the different peptidases in the different parts of the small intestine, so presumably more tetra- and tripeptidases will be absorbed in the proximal part and more dipeptidases and amino acids in the distal part. This means that the right form of the peptides needs to be in the region with the appropriate peptidases. (Caspary, 1992).

When the protein in milk, casein, is replaced or partly replaced by soybean flour, it has been seen that there is no curd formation in the abomasum, leading to a faster flow of milk through the abomasum. Consequently, less time is available for transport into epithelial cells of

peptides and amino acids because of the increased flow through the small intestine. This can lead to increased cases of osmotic diarrhea due to the missing curd formation (Gorrill and Thomas, 1967; Smith and Sissons, 1975). In three trials by Seegraber & Morril *et al.* (1986), Grant *et al.* (1989) and Montagne *et al.* (1999), they substituted part or all of the casein with soy flour or commercial soy protein concentrate. Montagne *et al.* (1999), who used heated soy flour, looked at differences in mucosa development in the small intestine compared to calves fed whole milk or regular milk replacer. Generally calves fed soy had more short, blunt and broad villi than the calves fed milk protein (Grant et al., 1989; Montagne et al., 1999; Seegraber and Morril, 1986). Seegraber & Morril (1986) found that, when using commercial soy protein concentrate the villi progressed to severe villous atrophy with intervillus bridging. Grant *et al.* (1989) found that soy protein concentrate tended to decrease mucosal protein as well as it might reduce efficiency of the epithelial protein synthesis in the small intestine mucosa. Montagne *et al.* (1999) found feeding soy depressed specific activity of a number of brushborder enzymes in the proximal jejunum. This is could be due to the trypsin inhibitor untreated soy contains, when the soy has been heat treated, the antitrypsin is inactivated. The trypsin inhibitor inhibits the effect of trypsin (Gorrill and Nicholson, 1971; Kakade et al., 1974). Trypsin is secreted from the pancreas as trypsinogen, the enzyme enteropeptidase splits of a part of trypsinogen and it becomes active trypsin, and activates other proteolytic enzymes secreted by the pancreas (Lu et al., 1997; Preiser et al., 1975).

In conclusion based on this chapter, the enzyme production in the young calf, of enzymes like chymosin and pepsin, is adapted for specifically digesting protein in milk the first weeks postnatally. Pepsin secretion increases rapidly around the time the calf becomes a ruminant which could suggest introducing other protein sources in starter grain could have an effect on the development of chief cells and mucous cells to start secreting pepsin. Furthermore, it can be concluded that replacing milk protein or some of the milk protein by soy flour or soy concentrate, effects villus development, epithelial protein synthesis and brushborder enzymes negatively.

### 2.4.2 Carbohydrates

From birth, up until the calf starts eating starter grain and is weaned, the calf has difficulties digesting other carbohydrates than lactose. The only carbohydrates that the calf can utilize from the MR is lactose, glucose and galactose. This is partly due to the low activity of maltase, sucrose and amylase and the high activity of lactose. The highest lactase activity is found in calves one week after birth, thereafter the lactase activity decreases over time but also depends

on weaning and earlier weaned calves have lower lactase activity than later weaned calves. Around 6-8 weeks, postnatally lactase activity has halved compared to at birth and around this time, maltase activity has doubled. Maltase activity, unlike lactase activity, increases with age, it reaches peak activity around 20 weeks of age (Coombe and Smith, 1974, 1973; Le Huerou et al., 1992; Siddons et al., 1969; Toofanian et al., 1973). Lactase is a brush-border enzyme which hydrolyses the lactose in milk to D-glucose and D-galactose, which are then transported across the intestinal epithelium by the $Na^+$ dependent glucose transporter 1 (SGLT1) (Dyer et al., 2003; Shirazi-Beechey et al., 1991). Amylase secreted from the pancreas increases with age, so as the calf becomes a ruminant, amylase activity increases and this enables the calf to digest starch from the feed (Siddons, 1968; Suárez et al., 2006a).

Feeding different levels of lactose in specially formulated MR showed no significant difference between the treatment groups and the control groups, on body weight gain during the trial and final empty bodyweight, even though one trial investigated different lactose and fat contents and the other trial investigated substituting part of the lactose by whey, corn syrup and/or dextrose (Bernard et al., 2013; Tikofsky et al., 2001). The MR was formulated according to crude protein (CP) (all-milk-derived) and fat (choice white grease), with; low fat (14.79% DM)– high lactose (55.29% DM), medium fat (21.62% DM) – medium lactose (46.69% DM) or high fat (30.62% DM) – low lactose (35.36% DM). There were eight calves in each group, treatments started when calves had reached an age between 2 and 6 days and were slaughtered at 85 kg body weight (Tikofsky et al., 2001). Or substituting part of the lactose in the milk replacer by other carbohydrates, keeping DM, CP (% DM), fat (% DM) and ash (% DM) the same between treatments, control group was fed a commercial MR. Treatments for formulated to contain; 24.5% whey, 48.7% whey protein and 41% lactose or 1.2% whey, 19.9% corn syrup solids, 53.3% whey protein and 25% lactose or 12.9% whey, 9.9% corn syrup solids, 51.5% whey protein and 33% lactose or 1.2% lactose whey, 9.9% corn syrup solids, 9.9% dextrose, 53.4% whey protein and 25% lactose. There were 20 calves in each group, treatments started when the calves reached an age of 3 days, commercial starter grain was offered ad libitum throughout the study, until weaning at 42 days of age. The trial ended at 56 days of age, calves were fed starter grain and water the last 14 days (Bernard et al., 2013).

A study by Gilbert *et al.* (2015) on 45 calves divided into 5 treatments; control and 4 treatments where either gelatinized starch, maltodextrin, maltodextrin with a high level of $\alpha$-1,6-branching or maltose was included in the milk replacer on expense of some of the lactose. The calves were introduced slowly to the treatments at 13 weeks of age and the experimental period began when they were 27 weeks of age and lasted for 3 weeks. The only source of

nutrients was 1 of the 5 MR's fed to the calves. They found that when adding gelatinized starch, calves had a higher mean daily body weight gain compared to control and maltose supplemented calves (Gilbert et al., 2015).

When looking at the carbohydrate sources and composition in the starter grain, some differences are seen in growth in terms of rumen and intestinal development and calf growth. A study by Hill *et al.* (2008) investigated the difference in calf growth when fed starter grain varying in the composition of carbohydrates fed to calves 3-4 days of age until 56 days of age. The starters fed were: low molasses; 5% molasses, 20% whole oats, 35% pellets and 40% rolled coarse corn, this were the control group or High molasses; 10 % molasses, 20% whole oats, 35%pellets and 35% rolled coarse corn or low molasses + sugar; starter 5 % molasses plus 1.5 % granular sugar, 20% whole oats, 35% pellets with sucrose and 40% rolled coarse corn. The pellets consisted of soybean meal, wheat middlings, alfalfa meal, minerals, vitamins and animal fat, in the pellet with sucrose 4.25 % sucrose was added. They found that the treatments had a higher average daily gain than the control group but there was no difference in gain to feed intake. They did find a tendency, of the calves in the treatment groups to have a higher hip width than the control group (Hill et al., 2008). Another study by Kosiorowska *et al.* (2010) investigated the effect of feeding two different concentrates, offered to 32 calves from birth to slaughter at 38 or 56 days of age. A commercial traditional concentrate, high-starch (350 g/kg DM), low-fiber (136 g NDF/kg DM), low-molasses (67 g sugar/kg DM) were fed or an experimental concentrate, low-starch (107 g/kg DM), high-fiber (296 g NDF/kg DM), high-molasses (140 g sugar/kg DM) were fed. The concentrates were combined with either a high amount of whole milk (2x3,2 kg) or a low amount of whole milk (2x1,6 kg). There were no significant differences between traditional concentrate fed group and experimental concentrate fed group when looking at final body weight, papillae development, jejunum villus height and crypt depth and empty body weight. But they did find a tendency of the traditional concentrate fed group to have a higher average daily gain than the experimental concentrate fed group and the experimental concentrate fed group had a significantly longer small intestine than the traditional concentrate fed group (Kosiorowska et al., 2011).

A study by Suárez *et al.* (2006) looked at rumen development, animal performance and rumen fermentation characteristics when feeding concentrates differing in carbohydrate composition. 160 calves, entering the study with an average bodyweight of 44.9 kg and slaughtered at an age of 8 or 12 weeks. Calves were divided between 5 dietary treatments; 1) milk replacer, the control group, 2) Pectin based concentrate (starch: 22.4 g/kg DM; sugar: 64.8 g/kg DM; NDF: 340 g/kg DM), 3) NDF-based concentrate (starch: 107.9 g/kg DM; sugar: 25.8

g/kg DM; NDF: 493 g/kg DM), 4) starch based concentrate (starch: 592.8 g/kg DM; sugar: 27.7 g/kg DM; NDF: 94 g/kg DM) and 5) mixed concentrate (starch: 243.9 g/kg DM; sugar: 35.2 g/kg DM; NDF: 308 g/kg DM). They found that the treatments compared to the control group, promoted rumen development and they all had higher total VFA concentrations in the rumen fluid. This is in agreement with the findings in earlier sections that concentrates are needed in order for the rumen to develop. Acetate showed to have the highest proportion followed by propionate and butyrate. When comparing the treatments, starch had a lower total VFA concentration in rumen fluid. But starch based concentrate fed calves showed the highest molar proportion of propionate and the molar proportion of butyrate was higher for calves fed the starch based concentrate, pectin based concentrate and mixed concentrate compared with the control group, fed milk replacer and the NDF-based concentrate fed calves (Suárez et al., 2006a, 2006b).

It has been shown that the digestive tract of young calves is designed to only digest carbohydrates in milk in the form of lactose. Around the time the calf starts eating starter grain and becomes a ruminant, maltase activity increases and amylase activity increases with age as well, which means as the calf grows older, it can digest other sources of carbohydrates. Differing the lactose level in MR does not alter growth of calves but when different carbohydrate sources in starter grain are evaluated, there is a difference between the types of carbohydrates. It was seen that starter grain with more sugar or starch, promoted calf growth by increased average daily gain. Rumen growth was positively affected by concentrate feeds compared to only milk.

### 2.4.3 Fatty acids

As mentioned in the chapter about protein, chymosin induces coagulation of ingested milk which binds casein and fat in the formed curd. This ensures a slow release of protein and fat to the small intestine (Longenbach and Heinrichs, 1998; Miyazaki et al., 2009; Yvon et al., 1984). The lipids in cow milk are approximately 50% long chained fatty acids but are rich on medium and short chain fatty acids, fatty acids with $<C_{16}$ , compared to milk from non-ruminants (Jensen, 2002; Soyeurt et al., 2006).

The fatty acids in the milk are broken down in the abomasum to triglycerides, free fatty acids, diglyceride and monoglycerides by the salivary lipase (Edwards-Webb and Thompson, 1978; Gooden and Lascelles, 1973). The salivary lipase is unable to lipolyse long chain fatty acids ($>C_{14}$). The pancreatic lipase can lipolyse long -, medium – and short chain fatty acids in contrast to the salivary lipase. The pancreatic lipase secretion is relatively small at calving but increases with age (Christi and Vadodaria, 1998). The most rapidly released fatty

acid by both the salivary and pancreatic lipase was butyric acid (Edwards-Webb and Thompson, 1977; Grosskopf, 1965). Pancreatic lipase released the longer chain fatty acids at rates one-third to half of butyrate (Edwards-Webb and Thompson, 1977). The short chain fatty acids butyric ($C_4$) and caproic ($C_6$) acid is esterified almost entirely at the sn-3 position from the milk fat, salivary lipase seems to have a preference for the sn-3 position, therefore the milk is a rich source of butyrate for the calf (Edwards-Webb and Thompson, 1977; Månsson, 2008). The mentioned salivary lipase released butyrate most rapidly. Butyrate was then followed by another SCFA, caporate. At a slower but fairly constant rate, other short and medium chained fatty acids ($C_8$ – $C_{12}$) were released. Acids with $C_{14}$ or longer chain lengths were released very slowly (Edwards-Webb and Thompson, 1977). In several trials, it was found that in both calves and goat kids, the salivary lipase decreases and disappears with increasing age (Grosskopf, 1965). When the fatty acids leave the abomasum of the pre-ruminant calf, approximately half of the lipids are leaving as triglycerides, approximately one quarter as free fatty acids, less than one quarter as diglyceride and a relatively small part as monoglycerides. (Edwards-Webb and Thompson, 1978; Gooden and Lascelles, 1973).

In a study by Hill et al. (2011), calves were fed a MR either supplemented with NeoTec 4 or not supplemented. NeoTec 4 is a blend of Butyrate, coconut oil and flax seed. The study was looking at how fatty acid intake alters growth and immunity in milk fed calves. The calves were 2-5 days of age when entering the trial and 56 days of age when ending the trial, there was 24 calves in each group. This meant that the calves supplemented with NeoTec had a higher percentage of short- and medium – chained fatty acids. NeoTec supplemented calves had a fatty acid intake of C4, C10 and C12 that was 14 times higher and C6 intake that was 7 times higher than calves not supplemented. C18:2 intake was almost the same and C18:3 was doubled. They found that calves fed more medium and short chain fatty acids (>$C_{16}$), the NeoTec supplemented calves, had a significantly higher body weight by the end of the study and body weight gain (kg/d) during the trial, compared to calves not supplemented. The calves supplemented with NeoTec also had a significantly higher MR intake (kg/d), thereby they also had a significantly higher intake of $C_4$, $C_{10}$, $C_{12}$, $C_{14}$ and $C_{18:3}$ fatty acids compared to calves not supplemented with NeoTec (Hill et al., 2011). Calves not supplemented with NeoTec had a significantly higher fecal score which means the feces was more thin in calves not supplemented compared to calves supplemented with NeoTec (Hill et al., 2011). From these results, it was concluded that supplementing milk replacer with more short and medium chain fatty acids can alter calf growth positively (Hill et al., 2011).

In a study by Vidyarthi & Kurar (1995) three groups of calves were compared. The three groups were fed whole milk, skim-milk, calf-starter and green fodder, the control group was fed this, with no supplementation. Another group was supplemented with butyric and propionic acid in a 1:1 ratio with gradually increasing amounts from 1 ml/d to 24 ml/d. The third group was supplemented with acetic, butyric and propionic acid in the ration of 1:1:1 with the same gradual increase as the butyric and propionic supplemented calves. The calves were in the trial from 11 days of age until euthanizing them at 60 days of age, with 6 calves in each group. They found that the supplementation with butyric and propionic acid seemed to be the supplementation with the best stimulatory effect on GIT development in buffalo calves. Supplementation with butyrate and propionate gave significantly higher GIT weight, bigger rumen weight and rumen expressed as % of total GIT, higher rumen volume, higher weight of the stomachs, higher rectum weight, more and longer papillae. The small intestine weight was significantly smaller in the acetic, butyric and propionic acid supplemented calves compared to the butyric and propionic acid supplemented calves and not supplemented calves (Vidyarthi and Kurar, 1995).

These trials indicate that feeding more short and medium chain length fatty acids can alter growth by promoting rumen development. It is also clear, based on this chapter, that the young calves' digestive tracts are designed to digest triglycerides with high content of short and medium chained fatty acids and hence absorb and metabolize these specific fatty acids as well. Based on the previous chapters, it is worth looking into SCFA supplementation in milk for calves, since protein and carbohydrates supplemented in milk did not seem to have an effect on rumen development and growth. However, in calves supplemented sources of sugar or starch and euthanized after weaning, growth seemed to be positively affected, possibly by fermenting carbohydrates to SCFAs.

## 2.5 Butyrate supplementation to the calf

As mentioned in the previous chapter, the calves' digestive tracts as a preruminant is specifically designed to digest fat containing short and medium chain length fatty acids. One of these fatty acids is butyrate which is also one of the SCFAs produced in the rumen during fermentation of carbohydrates (Guilloteau et al., 2010). Feedstuffs containing a high concentration of sugar, such as molasses, yields a relatively high proportion of butyrate as the end product compared to other carbohydrates (Hackmann et al., 2013; Kristensen et al., 2003).

In calves fed diets differing in carbohydrate composition, it has been seen that the carbohydrate sources that have the highest fermentation to butyrate as percentage or $\mu$mol/L also seemed to stimulate rumen development more positively than other SCFAs from other carbohydrate sources (Khan et al., 2008; Suárez et al., 2006b). Calves in the trial by Khan et al. (2008) were 3 days old when entering the trial and 70 days when ending the trial, they were fed four different diets differing in starch source, so the diet either contained ground barley, ground corn, crimped oats or wheat. The DM in these diets were similar, crude protein, starch and ash were kept similar as well. Calves in the trial by Suárez et al. (2006b) entered the trial at a mean weight on 44.9 kg and ended the trial when either 8 or 12 weeks old, they were fed either a pectin diet containing 91.3% ground beetpulp, 1.5% soybean oil, or NDF containing 46.4% soybean hulls and 46.4 % corn grits, or starch containing 46.4% corn and 46.4% crushed barley or mixed containing 30.3% ground beet pulp, 15.5% soybean hulls, corn grits, corn and crushed barley and 0.5 % soybean oil, the diets had similar crude fat, crude protein and DM. Rumen empty weight is higher in calves with significantly highest butyrate VFA concentration in the rumen, these calves were pectin and starch calves in the trial by Khan et al (2008) and corn and wheat calves in the study by Suárez *et al.* (2006b). Mucosa thickness is higher (Suárez et al., 2006b) and rumen wall thickness, papillae length, papillae width and papillae number/cm$^2$ were also higher in the calves with significantly highest butyrate VFA concentration (Khan et al., 2008). This could indicate that butyrate plays a particularly important role in the development of the rumen mucosa in calves. It has been shown when comparing infusion into the rumen two times a day of calves starting when the calves were 14 to 35 days old and the calves were slaughtered after 11 weeks at an age of 73 to 109 days of age. They were infused with either Na-butyrate, Na-acetate, Na-propionate, glucose, Na-chloride or nothing, butyrate promoted the best papillary development. Butyrate infused calves had a significantly higher percent mucosa of rumen tissue and significantly higher rumen weight. Butyrate and propionate infused calves had significantly higher mucosa weight, compared to the other treatments (Sander et al., 1959). Even though the treatment groups in the trials by Sander *et al.* (1959) were consisting of only 2 calves per group, further research with butyrate has been done when comparing whole milk to MR and MR supplemented with sodium butyrate, feeding whole milk gives the overall best effect on rumen development. This was in a trial with 7 calves per group, the calves entered the trial at an age of 5±1 days of age and the trial lasted for 21 days, DMI was kept similar and constant over the trial (Górka et al., 2011b). When looking at apoptotic index MR + sodium butyrate has significantly lowest index and a significantly higher sum of VFA and acetate and propionate concentration (Górka et al., 2011b). It has also been seen that rumen weight is significantly

positively correlated with small intestine weight and activity of brush border enzymes; lactase, maltase, aminopeptidase A, aminopeptidase N (Górka et al., 2011b). If butyrate promotes rumen growth, there could be a correlation to intestinal development in calves. In the chapter, focus will be upon 7 research papers investigating the effect of supplementing different forms of butyrate on the growth and development of the calf and its GIT.

### 2.5.1 Calf growth

It has been possible to find 7 different trials, where the effect of adding butyrate in the milk replacer fed to calves versus no supplementation of butyrate were investigated on growth, development of the intestines and/or rumen and health Araujo *et al.* (2013), Gorka *et al.* (2009), Górka *et al.* (2014), Górka *et al.* (2011), Guilloteau *et al.* (2009), Kato et al., (2011) and Nazari *et al.* (2012). In these studies, butyrate was added to the MR for young calves from 3-12 days of age and for durations of 21-139 days. The added butyrate was added either in the form of sodium butyrate, Tributyrin or calcium coated butyrate. In the trials by Górka *et al.* (2011) and Górka *et al.* (2014), the calves could also be offered butyrate in the starter grain. The calf could either receive MR and starter grain supplemented with butyrate in both MR and starter grain or in either MR or starter grain or no supplementation in MR or starter grain.

In table 2.1, the methods from the 7 trials are summarized. The table shows how many calves per group, the sex and breeds of the calves, what age the calves were at the beginning of the trial, how long the trial lasted, how many times a day the calves were fed milk, if the amount of ingested milk was recorded, it is also included and the amount of butyrate added in the MR or starter grain. The 7 different trials all kept records on body weight and starter grain intake which were used for calculations of average daily gain (ADG) and feed conversion ratio. These results are also summarized in table 2.1. In three of the trials, either Body Condition Score (BCS) or body frame measurements were used as well to monitor growth of the calves, these results are included in table 2.1.

Górka *et al.* (2011) and Górka *et al.* (2014) show results from the same trial but Górka *et al.* (2011) focus on growth, health and rumen development, whereas Górka *et al.* (2014) focus on intestinal development and enzyme activity. Therefor their methods are summarized together.

Studies have found that when preruminant calves are supplemented with butyrate in the MR, they have a significantly higher (Guilloteau et al., 2009b; Nazari et al., 2012), tendency of a higher (Gorka et al., 2009; Górka et al., 2011a), or numerically higher (Kato et al., 2011) ADG, but it seems that some of the calves also consume more MR to have this higher

ADG (Górka et al., 2011). If this increased ADG has a positive effect later on for the milk production potential of the cow, it might pay off to supplement butyrate and get a higher ADG even though the calves consume more. Long-term effects of accelerated growth as a calf will be discussed later in chapter 2.5.6. Other trials show that calves with a higher ADG consumes less feed, maybe not per day but until weaning and thereby has a significantly or numerically lower feed conversion ratio (Feed intake/ADG) (Gorka et al., 2009; Guilloteau et al., 2009b; Kato et al., 2011; Nazari et al., 2012). One trial found that ADG was significantly decreased when feeding Tributyrin. This might be due to the form of butyrate since in the same trial they recorded no significant difference between control and sodium butyrate supplemented calves (Araujo et al., 2015).

It was shown that supplementing butyrate either had no effect (Górka et al., 2011a) or a tendency (Gorka et al., 2009) of an effect on body condition scoring, increasing body condition score. This is also seen when measuring body frame, rump height, wither height and hip width. It was found that at day 27 of age and weaning at day 51 calves supplemented with butyrate had higher body frame measurements compared with control (Nazari et al., 2012). The reason for the variation in the significance of differences between calves supplemented with butyrate and control looking at body condition score and body frame measurements may be due to the two different ways of measuring a change in the growth of the calves. Body condition score measures more of a difference in fat deposits in specific regions subcutaneously, this is not necessarily related to intra-abdominal fat deposits. Body frame measurements measure the actual bone growth of the calf and is not influenced directly by the fat deposits.

**Table 2.1. Methods and calf growth; calf information, milk feeding, starter grain, butyrate supplementation, average daily gain, feed:gain ratio, BCS & Body frame measurement**

| Groups | Guilloteau *et al.* (2009) | | Nazari *et al.* (2012) | | Kato *et al.* (2011)[1] | | Górka *et al.* (2011) & Górka *et al.* (2014)[2, 3] | | | | Araujo *et al.* (2015) | | | Górka *et al.* (2009)[4,5] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Flavomycin | Na-butyrate | Control | Coated calcium butyrate | Control | Na-butyrate | $MR^-/SM^-$ | $MR^-/SM^+$ | $MR^+/SM^-$ | $MR^+/SM^+$ | Control | Tributyrin | Na-Butyrate | Control | Na-Butyrate |
| n | 44 | 44 | 8 | 8 | 12 | 12 | 7 | 7 | 7 | 7 | 17 | 17 | 17 | 7 | 7 |
| Sex & breed | Male Holstein | | Female Holstein | | Male Holstein | | Male Holstein or Holstein x Limousine | | | | Holstein | | | Male Holstein | |
| Trial period (d) | 139 | | 48 | | 42 | | 21 | | | | 42 | | | 21 | |
| Age, entering trial (d) | 12 | | 3±1 | | 3±1 | | 5±1 | | | | 11±3.1 | | | 4-6 | |
| Milk feedings/day | 2, Sunday only 1 | | 3 | | 2 | | 2 | | | | 2 | | | 2 | |
| Milk | | | 4 L/d | | | | 10% of initial BW | | | | 4 L/d at 15% DM | | | 10% of initial BW | |
| Butyrate amount | MR contained 0.3% of DM | | 3 g. Coated calcium butyrate pr. total daily milk allowance | | Na-butyrate: 3 g/d, day 1 to 3 5 g/d, day 4 to 7 7 g/d, day 8 to 42 | | [6]$SM^+$: 0.6% Na-butyrate encapsulated within triglyceride matrix as fed $MR^+$: 0.3% crystalline Na-butyrate as fed | | | | Tributyrin at 0.3% DM Na-butyrate at 0.3 % DM | | | 0.3% as fed crystalline Na-butyrate | |
| starter grain | Ad libitum | | Ad libitum | | Ad libitum | | Ad libitum | | | | Ad libitum | | | Ad libitum | |
| ADG[7] (g/d) | 1139±14[a] | 1177±14[b] | 457.6±29.8[a] | 609.9±15.5[b] | 654±31 | 710±26 | 14[e] | 36[e] | 88[f] | 95[f] | 530[a] | 420[b] | 480[ab] | 16±19[e] | 95±35[f] |
| Feed:gain ratio | 1.54±0.02[a] | 1.48±0.01[b] | 1.38±0.053[a] | 1.05±0.037[b] | 0.634 | 0.564 | 2.0 | 2.7 | 1.9 | 2.4 | 0.57 | 0.72 | 0.63 | 5.94 | 1.43 |
| BCS[8] | | | | | | | no differences | | | | | | | 4.84±0.06[e] | 4.94±0.04[f] |
| Body frame measurement | | | | increased rump height, wither height & hip width | | | | | | | | | | | |

[a, b] *Within the same column and study not bearing a common subscript differ (P≤0.05)*

[e, f] *Within the same row and study not bearing a common subscript has a tendency of differing in results (P≤0.10)*

[1] *Mean ADG calculated from kg from d. 4-43 starter intake and average daily weight gain*

[2] *$MR^+$ is Milk replacer supplemented with butyrate, $MR^-$ is milk replacer not supplemented with butyrate, $SM^+$ is starter grain supplemented with butyrate, $SM^-$ is starter grain not supplemented with butyrate.*

[3] *ADG is given as g. DMI/kg BW*

[4] *Górka et al. (2009) declared tendency of a significant difference at P<0.15, and significant difference at P<0.10, to compare with the other articles, tendency is declared at P<0.10 and significance is declared at P<0.05.*

[5] *feed conversion ratio calculated from starter grain intake and average daily gain*

[6] *Supplemented with Na-butyrate, encapsulated within triglyceride matrix. 0.6% as fed, 30:70 butyrate to triglyceride matrix*

[7] *Average daily gain*

[8] *Body condition score 5-point scale - 1 – bad, 5 – very good*

*scale - 1 – bad, 5 – very good*

### 2.5.2 Rumen growth

Out of seven different articles, three of them focused on rumen development. Table 2.2 summarizes their results for weight of rumen, omasum and abomasum relative to the whole stomach weight.

Calves supplemented with butyrate in MR have been seen to have either significantly (Gorka et al., 2009; Górka et al., 2011a) or numerically (Kato et al., 2011) bigger rumen expressed as percent of the whole stomach. Calves supplemented with butyrate in the starter grain seem to be affected by the butyrate as well, since there is a tendency of those calves having a larger (as percent of the whole stomach) rumen than calves not supplemented in the starter grain (Górka et al., 2011a). It seems when supplementing butyrate in either milk replacer or starter grain, the rumen is proportionately increasing in size, as percent of whole stomach weight, the abomasum is proportionally decreasing in size, as percent of whole stomach weight, whereas omasum seems to be relatively proportional constant and not affected by rumen or abomasum size when expressed as percent of whole stomach weight. But that does not mean that the omasum is not growing, the absolute volume could be increased. (Gorka et al., 2009; Górka et al., 2011a; Kato et al., 2011). Butyrate has been found to have a positive effect on the rumen development, more specifically the papillae development in the rumen cranial ventral and dorsal sac. When calves are supplemented with butyrate in the milk replacer, papillae are longer and in the cranial dorsal sac also wider than in calves not supplemented. These results were either found significantly or as a tendency(Górka et al., 2011; Pawel Gorka et al., 2009). This in agreement with the above mentioned relatively bigger rumen in supplemented calves. One of the studies did not find a significant neither a tendency of a significant effect of supplementing butyrate to calves on rumen, omasum and abomasum size, nor papillae length, only a numerical difference was seen showing the same as the results mentioned above (Kato et al., 2011). These results could indicate that an increase in butyrate supplementation from 3 g/d to 5 g/d to 7g/d does not have a positive effect on the weight of the rumen -, omasum -, abomasum - weight or rumen papillae length compared to a constant amount of butyrate supplemented (Kato et al., 2011). The fact that the calves in the study were older at the time of slaughter may mean those calves have eaten more starter grain and have better developed rumen and starts to ruminate and ferment the feed. This may also have an effect on the results since calves fermenting carbohydrates in the rumen, produces more SCFA, thereby more butyrate, stimulating papillae growth.

**Table 2.2 Rumen development, whole stomach weight, rumen, abomasum and omasum % of whole stomach weight, papillae length and width**

| Groups | | Kato *et al.* (2011) | | Górka *et al.* (2011) | | | | Górka [1] *et al.* (2009) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | Na-butyrate | $MR^-/SM^-$ | $MR^-/SM^+$ | $MR^+/SM^-$ | $MR^+/SM^+$ | Control | Na-butyrate |
| Whole stomach weight (g)[2] | | 1496±46 | 1475±79 | 670 | 777 | 734 | 736 | 622±59 | 728±36 |
| Reticulorumen (% of total stomach weight) | | 59.7±2.0 | 60.4±2.8 | $43^{a, e}$ | $49^{a, f}$ | $47^{b, e}$ | $50^{b, f}$ | $42.6±2.3^{a}$ | $49.8±1.1^{b}$ |
| Omasum (% of total stomach weight) | | 14.2±1.2 | 13.3±1.1 | 12 | 12 | 11 | 11 | 11.9±0.6 | 10.6±0.4 |
| Abomasum (% of total stomach weight) | | 26.1±1.3 | 26.5±1.3 | 45 | 39 | 42 | 39 | $45.5±2.4^{a}$ | $39.5±1.2^{b}$ |
| papillae length (μm) | Cranial ventral sac | 2240±230 | 2540±350 | $600^{c}$ | $787^{c}$ | $932^{d}$ | $856^{d}$ | | |
| | Cranial dorsal sac | | | $314^{a, e}$ | $457^{b, e}$ | $443^{a, f}$ | $516^{b, f}$ | $314±16^{a}$ | $516±34^{b}$ |
| papillae width (μm) | Cranial ventral sac | | | 181 | 302 | 302 | 277 | | |
| | Cranial dorsal sac | | | $150^{a, c}$ | $185^{b, c}$ | $186^{a, d}$ | $228^{b, d}$ | $150±8^{a}$ | $228±12^{b}$ |

*[1] Górka et al. (2009) declared tendency of a significant difference at P<0.15, and significant difference at P<0.10, to compare with the other articles, tendency is declared at P<0.10 and significance is declared at P<0.05.*

*[2]Górka et al. (2011) is calculated from whole stomach % of BW*

*[e, f] within the same row and study not bearing a common subscript has a tendency of differing in results (P≤0.10)*

*[a, b, c, w]ithin the same row and study not bearing a common subscript differ*

### 2.5.3 Intestinal growth

Out of seven different publications, two of them focused on butyrate supplementations effect on small intestinal development. They looked at the small intestine length, villus height, crypt depth and villus height to crypt depth ratio, and from the various parts of the small intestine; duodenum, jejunum and ileum. The results are summarized in table 2.3.

Supplementation of butyrate in the milk replacer generally tends to have a positive effect on duodenum development. It has been seen that the duodenum is longer in supplemented calves. This result has been seen both numerically (Górka et al., 2014) and as a tendency (Guilloteau et al., 2009b). When looking at villus height and crypt depth, the results are contradictory. When calves are only supplemented with butyrate in the MR, villus height is significantly higher and crypt depth are numerically deeper in duodenum in supplemented calves compared to control (Guilloteau et al., 2009b). When calves were supplemented with butyrate in the starter grain only, crypt depth is significantly increased in duodenum (Górka et al., 2014). It seems like butyrate is less effective in stimulating duodenal growth when it is supplemented in both starter grain and MR (Górka et al., 2014). In the jejunum, the effect of butyrate supplementation on development is different depending on region of the jejunum. When calves are either only supplemented in the milk replacer or starter grain, jejunum was seen to be numerically and significantly absolutely shorter (Górka et al., 2014; Guilloteau et al., 2009b). Supplementing butyrate in MR has a negative effect on villus height in proximal and middle jejunum and on crypt depth in middle jejunum. Villus height in Distal jejunum and crypt depth in proximal and distal jejunum is either significantly or numerically positively affected by only supplementing butyrate in the milk replacer or by supplementing in the starter grain either in combination with supplementation in MR or not (Górka et al., 2014; Guilloteau et al., 2009b). When looking at Ileum development, results are contradictory. In a trial only supplementing butyrate in the milk replacer and calves are euthanized at 26 days, before they become ruminants, Ileum length and ileum crypt depth are numerically lower, but ileum villus height numerically higher (Guilloteau et al., 2009b). Whereas in another trial when butyrate is supplemented in both MR and starter grain or in one of them results are different, when looking at the length of the Ileum supplementing in both MR and starter grain has the most positive effect on the length, but when looking at villus height and crypt depth, villus are numerically higher in calves supplemented with butyrate in the milk, whereas crypt depth seems to be highest in calves not supplemented at all (Górka et al., 2014). The differences between control calves and supplemented calves in the study by Guilloteau *et al.* (2009) compared to Górka *et al.* (2014) might be due to the difference in age when the calves are euthanized. Górka et al. (2014)

euthanized the calves when they were 151 days old and the calves had developed into ruminants. Whereas Guilloteau *et al.* (2009) euthanize their calves when they are 26 days old, at this point the calves are still on milk and the rumen has only just begun to develop. This means that the calves in the study by Górka *et al.* (2014) have started to ferment carbohydrates and SCFA's are being produced by microbes in the rumen, so they might have a higher amount of butyrate reaching the intestines.

**Table 2.3. Small intestine length, villus height, crypt depth and villus height to crypt depth ratio**

| | Guilloteau *et al.* (2009) | | Górka *et al.* (2014) | | | |
|---|---|---|---|---|---|---|
| Groups | F | B | $MR^-/SM^-$ | $MR^-/SM^+$ | $MR^+/SM^-$ | $MR^+/SM^+$ |
| **Small intestine length** | | | | | | |
| Total (m) | | | $13.8^a$ | $12.0^b$ | $12.4^c$ | $14.5^d$ |
| duodenum (cm) | $58.2\pm3.5^e$ | $65.1\pm1.3^f$ | 32 | 28 | 33 | 33 |
| jejunum (cm) | 1840±60 | 1830±90 | $1310^a$ | $1150^b$ | $1180^c$ | $1360^d$ |
| Ileum (cm) | 79.1±5.0 | 74.5±6.5 | $39^{a, b}$ | $25^b$ | $35^{a, b}$ | $57^a$ |
| **villus height (µm)** | | | | | | |
| duodenum | $649\pm64^a$ | $869\pm111^b$ | 540 | 633 | 558 | 530 |
| Proximal jejunum | 1133±144 | 1115±68 | $651^a$ | $723^a$ | $626^b$ | $579^b$ |
| Middle jejunum | 944±301 | 788±111 | $392^a$ | $454^a$ | $353^b$ | $334^b$ |
| Distal jejunum | 674±118 | 848±175 | $512^a$ | $670^b$ | $574^a$ | $611^b$ |
| Ileum | 628±32 | 664±53 | 410 | 443 | 487 | 461 |
| **crypt depth (µm)** | | | | | | |
| duodenum | 277±27 | 279±24 | $257^b$ | $368^a$ | $305^b$ | $284^b$ |
| Proximal jejunum | $265\pm24^a$ | $291\pm24^b$ | 245 | 271 | 245 | 279 |
| Middle jejunum | $305\pm26^a$ | $258\pm19^b$ | $179^a$ | $197^a$ | $159^b$ | $132^b$ |
| Distal jejunum | $274\pm32^a$ | $352\pm54^b$ | 512 | 670 | 574 | 611 |
| Ileum | 259±13 | 244±16 | $289^b$ | $223^a$ | $247^{a, b}$ | $249^{a, b}$ |
| **villus:crypt ratio** | | | | | | |
| duodenum | | | $2.10^a$ | $1.73^b$ | $1.84^{a, b}$ | $1.86^{a, b}$ |
| Proximal jejunum | | | $2.68^a$ | $2.69^a$ | $2.55^b$ | $2.11^b$ |
| Middle jejunum | | | 2.21 | 2.30 | 2.43 | 2.59 |
| Distal jejunum | | | 2.12 | 2.74 | 2.57 | 2.78 |
| Ileum | | | 1.42 | 1.98 | 1.97 | 1.85 |

$^{a, b, c, d}$*Within the same column and study not bearing a common subscript differ (P≤0.05)*

$^{e, f}$*Within the same row and study not bearing a common subscript has a tendency of differing in results (P≤0.10)*

### 2.5.4 Health scores

In three of the trials, they looked at health parameters and used scoring, the results are summarized in table 2.4 for fecal consistency, scouring days and days on electrolytes.

There is a tendency for calves supplemented with butyrate in milk replacer or in starter grain to have a better fecal consistency, meaning the consistency is more thick and normal and they also had a tendency of having less days on electrolytes in the milk fed period (Gorka et al., 2009; Górka et al., 2011a). Butyrate does have an effect on the health of the calves as well as the growth. Both in calves supplemented butyrate in the MR and in the starter grain, there is a significant effect of supplementing butyrate on days with scours, calves supplemented have significantly less days with scours compared to calves not supplemented (Gorka et al., 2009; Górka et al., 2011a). This could show a tendency of butyrate being effective in preventing and/or fighting scours in calves.

**Table 2.4 Fecal consistency, days with scour and days on electrolytes**

| | Górka et al. (2011) | | | | Górka [5] et al. (2009) | |
|---|---|---|---|---|---|---|
| Groups | $MR^-/SM^-$ | $MR^-/SM^+$ | $MR^+/SM^-$ | $MR^+/SM^+$ | Control | Na-Butyrate |
| Fecal consistency[2] | $1.32^e$ | $1.29^f$ | $1.39^e$ | $1.20^f$ | $1.39\pm0.09^e$ | $1.17\pm0.06^f$ |
| scour (days) | $1.57^a$ | $0.83^b$ | $1.86^a$ | $0.14^b$ | $1.57\pm0.61^a$ | $0.14\pm0.14^b$ |
| Electrolyte[1] (days) | $0.86^e$ | $0.67^f$ | $1.28^e$ | $0.00^f$ | $0.71\pm0.36^e$ | $0.14\pm0.14^f$ |

*[2]5-point scale: 1= normal, 2 = frothy, 3 = mucous, 4 = sticky, 5 = hard*

*[5] Górka et al. (2009) declared tendency of a significant difference at P<0.15, and significant difference at P<0.10, to compare with the other articles, tendency is declared at P<0.10 and significance is declared at P<0.05.*

*[a, b] Within the same column and study not bearing a common subscript differ (P≤0.05)*

*[e, f] Within the same row and study not bearing a common subscript has a tendency of differing in results (P≤0.10)*

*[1]Electrolytes given when fecal fluidity had a score of 3 or 4.*

Based on these seven articles, it can be concluded that calves supplemented with butyrate seem to have a higher ADG, body frame and BCS than calves not supplemented. Results were contradictory when looking at how much the calves ate to have a higher ADG. Supplementing butyrate had a positive effect on rumen development in rumen size, papillae length and width. Results on intestinal length, villus height and crypt depth were contradictory and few results were significantly affected by butyrate supplementation in milk replacer, therefore more research is needed on the effect of butyrate on small intestinal development. From this, it can be

concluded that supplementing 3 g. of sodium butyrate in the milk replacer fed two times a day has been seen to have a positive effect on rumen development and calf growth.

### 2.5.5 Long-term effects of supplementing butyrate

As it has been reviewed in the past chapters, supplementing butyrate in the milk replacer can have an effect on the calf in form of accelerated growth, compared to not supplementing butyrate. This chapter will review what effect accelerated growth, in form of bodyweight and GIT weight, means for future milk production for the cow.

Several trials kept records of ADG and weaning weight for calves, receiving different treatments, investigating different feeding strategies, and MR versus whole milk. Common for these trials with different methods was that the calves with highest ADG and weaning weight in most cases will have close to same heifer ADG, if not lower than the calves compared to. They will have around same calving weight with small variation compared to calves with the lower calf ADG and weaning weight (Bar-Peled et al., 1997; Moallem et al., 2010; Morrison et al., 2009; Shamay et al., 2005; Terré et al., 2009). Most importantly when they calve and become a part of the milking herd, most studies found no significant difference in milk yield when having accelerated growth as a calf (Morrison et al., 2009; Terré et al., 2009). One found a tendency of the calves with the higher growth as calves to have higher milk yield (Bar-Peled et al., 1997) and two studies found that the calves with a significantly higher weaning weight had a significantly higher milk yield (Moallem et al., 2010; Shamay et al., 2005). This suggests that the accelerated growth induced by butyrate may not have a negative effect on future milk production and may even give a higher milk yield.

## 2.7 Summary

In this literature review, the calves' digestive system has been under investigation. It was seen that when a calf is born, it is born as a monogastric and the forestomachs are yet to develop. The first 24 hours, the intestine is "open" and capable of absorption of Ig, immune cells and various cytokines which is essential for the calf since it is born with a competent immune system and do not receive maternal Ig's in foetal life. These milk antibodies are proteins so the colostrum contains colostral trypsin inhibitor in order for the proteins to reach the small intestine intact for absorption. The esophageal groove leads the milk past the forestomachs so the milk can reach the abomasum where digestion of nutrients in the milk can begin to happen. The GIT has three major phases of development. Phase 1 with proliferation and morphogenesis of mucosal cells, phase 2 where cells differentiate and phase 3 maturation which begins to happen already in foetal

life and continue in the neonatal calf. There is no sharp time definition of the phases but phase 3 occurs mostly postnatally.

The preruminant calves' digestive system is designed to digest the nutrients in cow milk. Digestion of protein, carbohydrates and fat starts in the abomasum. Most protein is broken down to peptides in the abomasum by chymosin, prochymosin and pepsinogen, before entering the small intestine. Most of the carbohydrates is lead straight through the abomasum to the small intestine, but as with protein some fat starts its digestion in the abomasum due to chymosin that binds protein and fat to form a curd in the abomasum. This means that protein and fat stay in the abomasum longer and are slowly released to the small intestine. Lactose is the main carbohydrate for the calf, since lactase has a high activity in the young calf and amylase, maltase and sucrose have a very low activity. Carbohydrate source and protein in the milk do not seem to have a significant effect on rumen and intestinal growth. But increased starch in starter grain fed to calves pre- and post weaning, has shown to have a positive effect on calf growth. Milk contains both long, short and medium chained fatty acids, the salivary lipase has a preference for the sn-3 position, esterifying butyrate and caporate. The calves' digestive tract is designed to digest these short and medium chain length fatty acids and does not utilize long chained fatty acids to well.

SCFAs are naturally occurring end products from the fermentation of carbohydrates in the rumen of ruminants. Butyrate, one of these SCFA's is especially occurring as a fermentation end product when feedstuffs like molasses are used. SCFA's has been seen to have a positive effect on calf growth and GIT development. Trials investigating the supplementation of SCFA's and carbohydrate sources in ruminating calves found that butyrate seems to be the SCFA with the most positive effect on rumen growth and calf growth.

Calves supplemented with butyrate in the MR showed a higher ADG, but also seemed to consume more grain. But as described in section 2.5.6 this increased ADG might give a higher milk yield or at least milk yield is not affected by increased growth. It seems like when Tributyrin is used as a supplement in MR ADG is decreased. It seems like supplementing could potentially increase bone growth, measured through various body frame measurements. Butyrate shows a tendency of a positive effect on calf health, since days with scours and days on electrolytes were lower in supplemented calves. Calves supplemented with butyrate also showed an improved rumen development through increased rumen weight, papillae length and papillae width. The studies on intestinal development showed results not strong enough to support the hypothesis that intestinal growth is affected positively. Proximal and middle jejunum seemed to be affected negatively by butyrate supplementation, ileum seemed to be affected positively on

length and villus height, but negatively on crypt depth, only duodenum and distal jejunum seemed to be positively affected.

Further studies on butyrate supplementation in the calf is needed, different doses of butyrate have not been studied in milk replacer, would a higher supplementation of butyrate increase calf growth and health. What effect would another form of butyrate have, more specific monobutyrin. Also, the effect on intestinal growth is still unclear, and could butyrate supplementation have an effect on intestinal permeability and tight junction proteins. Could rumen development be affected by the amount of butyrate, even though milk passes through the rumen into the abomasum. This will be investigated in the research trial described in the next chapter.

# 3. Research project

## 3.1 Abstract

Butyric acid is a short chain fatty acid produced in the forestomach of ruminants by fermentation, and it is also a naturally occurring fatty acid in cow's milk fat. Supplementing sodium butyrate in milk replacer to preweaning calves has been seen to enhance growth performance and rumen development. The aim of this study was to determine whether feeding milk replacer with no monobutyrin, a low dose of monobutyrin or a high dose of monobutyrin would enhance preweaning calves' performance, health and growth. Twenty-two Holstein bull calves were divided into three treatment groups. CON group received milk replacer without monobutyrin, LOW group received milk replacer with a low dose (0.37% of DM) of monobutyrin and HIGH group received a milk replacer with a high dose (0.75% of DM) of monobutyrin, for 8 weeks, starting when the calves entered the trial at an age of ≤ 4 days. The calves were fed milk replacer morning and afternoon and had access to textured starter grain and water ad libitum. LOW was numerically higher in water intake, MR intake and starter grain intake compared to CON and HIGH, in some weeks this difference was seen significantly. Body weight was measured every week, and was numerically higher in LOW treatment group. The study showed that LOW had numerically higher body frame measurements compared to CON and HIGH. The results were not found significantly, but this might be due to the limited number of calves (n=22). Tissue samples were taken from middle jejunum and rumen cranial sac at euthanasia (~56 days of age). It was seen that CON had significantly longer papillae and a higher length to width ratio. Jejunum villus height to crypt depth ratio was significantly higher in LOW group compared to CON. When looking at the tight junction proteins, occludin and claudin-1 it was seen that LOW and HIGH were significantly higher. Based on these results it was concluded that a low dose of monobutyrin supplemented in milk replacer positively affected calf growth. However further studies are needed on the effect of monobutyrin supplemented in milk replacer to determine the effect on the rumen epithelial development and the jejunum epithelial development.

## 3.2 Introduction

Butyric acid is a short chain fatty acid (SCFA) naturally present in cow's milk fat (Månsson, 2008). Butyrate in particular sodium butyrate has been seen to have a positive effect on the immune defense, and it is therefore anticipated that supplementing butyrate in the milk replacer

to calves might improve their health (Guilloteau et al., 2010, 2009a). Feeding sodium butyrate to weaning piglets has been seen to promote growth performance and the development of the intestinal mucosa (Kotunia et al., 2004; Le Gall et al., 2009), however in a different study only a small stimulating effect on intestinal mucosa morphology was seen (Tonel et al., 2010). In piglets, these controversial results could be due to the amount of sodium butyrate supplemented. It has been seen in cell cultures that a high dose of butyrin compared to a low dose may disrupt mucosal barrier function. In cell cultures it was seen that with a high dose of butyrin there was an increased rate of apoptosis in the intestinal epithelial cells. (Peng et al., 2007).

In cattle, especially dairy cattle, increased development of the rumen and intestines and possibly bigger surface of the rumen and intestines can result in a better nutrient uptake, and thereby in the future possible higher milk yield. In calves supplemented with sodium butyrate, sodium butyrate has been seen to enhance growth performance of the calf & rumen development, possibly due to stimulation of epithelial cell proliferation, differentiation and decreasing apoptosis in the rumen of calves 26, 45 or 151 days of age, when samples from rumen were collected.(Górka et al., 2011a; Guilloteau et al., 2009b; Kato et al., 2011) The supplementation of Na-butyrate does also seem to have a positive effect on the health of calves (Górka et al., 2011a; Guilloteau et al., 2009b). In vitro supplementation of Na-butyrate shows an increase in tight junction proteins, which could mean a decrease in para-cellular permeability, this has not been studied extensively in vivo yet. (Wang et al., 2012) different doses of butyrate have not been studied extensively, Kato *et al.* (2011) increased Na-butyrate intake supplementation over time. Calves were 3±1 days old when entering the trial, butyrate was supplemented 3 g/d day 1 – 3, 5 g/d day 4-7 and 7 g/d day 8 to 42. In the study by Kato *et al.* (2011) the increase in supplementation did not give a significantly increased calf growth. In cell cultures, with the human colonic epithelial cell line Caco-2 cells a high dose (8 mM) of butyrin compared to a low dose (2 mM), could disrupt the mucosal barrier function. The high dose had an increased rate of apoptosis in the intestinal epithelial cells (Peng et al., 2007). This could indicate there is a maximum in how much butyrate to supplement in order for the butyrate supplementation to have a positive effect.

In this study, we hypothesized that a low (0.37% of DM) supplementation of monobutyrin in milk replacer fed to preweaned calves, would enhance small intestine epithelial development, rumen development, health and growth performance in preweaned neonatal calves. Thus, the aim of this study was to determine whether feeding milk replacer with no monobutyrin, a low dose of monobutyrin or a high dose of monobutyrin would enhance preweaning calves' performance, health and development of rumen and small intestine.

## 3.3 Materials and methods

### 3.3.1 Animals, diets and management

All procedures of animal experiment were approved by Institutional Animal Care and Use Committee at University of California, Davis (UCD), USA. The trial was conducted in the animal research facility at UCD from October to December 2016. Twenty-two Holstein bull calves (≤ 4 days of age) were purchased from a local commercial calf ranch and transported to the research facility at the same time. Based on arrangements with the farmer, all calves should be fed ~3.8 L of maternal colostrum within 12 h after birth. Upon arrival, calves were weighed and intramuscularly injected with antibiotic (4 ml. Bio-Mycin® 200, Boehringer Ingelheim). Blood samples were collected from a jugular vein by venipuncture to harvest serum. Status of passive immunity was assessed from serum samples using Brix refractometer, which has been suggested to be positively correlated with serum IgG and total protein concentrations (Deelen et al., 2014). This 8-week study was conducted as a completely randomized design. Calves were stratified by BW at arrival and status of passive immune transfer and randomly distributed to three treatment groups that differed in dose of monobutyrin supplementation to a milk replacer (MR), including 1) CON: without monobutyrin supplementation, 2) LOW: monobutyrin supplementation as 0.37% of DM, and 3) HIGH: monobutyrin supplementation as 0.75% of DM.

The calves were housed individually in calf hutches bedded with rice hulls from arrival to weaning at the end of week 8 after arrival. Milk replacer (customized non-medicated MR with milk components only, Calva Products Inc., Acampo, CA, USA) was reconstituted with warm water (~38 to 43°C) at the ratio of 13% to 87% (wt/vol) and offered at equal volumes twice daily (0630 and 1630 h) from week 1 to 7 and once daily (0630 h) on week 8. Monobutyrin was supplemented at each feeding by mixing with the liquid MR. The daily feeding rate of MR (powder basis) was 1.5% of BW from week 1 to 6. Then, weaning gradually started by feeding MR (powder basis) at 1.3% of BW during week 7 and 0.75% of BW during week 8. BW was measured at the end of each week, thus feeding volume of MR was updated based on the most recent BW of calves. Textured starter grain (Associated Feed & Supply Co., Turlock, CA) and water were offered ad libitum throughout the study.

Every time a new calf starter and milk replacer bag was opened, samples were taken. The nutrient composition of the starter grain and milk replacer based on pooled samples, is shown in table 1. SOP's, various data recording schedules, bodyframe measurement standards, health score standards and hematoxylin and eosin staining protocol can be found in appendix A.

### 3.3.2 Measurements and sample collection

The MR and starter samples were collected on weekly basis from different bags and saved at -20°C. Pooled sample of MR and starter grain were submitted to commercial lab for analysis of nutrient composition (Cumberland Valley Analytical Service, MD, Table 1). Intake of MR, starter and water were recorded daily. Health status was assessed once daily prior to the morning feeding by scoring for alertness (5 scales with 1 = alert, bright clear eyes, ears up and 5 = flat on the side, severe depression), fecal consistency (4 scales with 1 = firm, well-formed and 4 = liquid splatters; Pineda *et al.*, 2016), respiration (5 scales with1 = normal breathing and 5 = chronic dry cough, weak to rapid breathing), and nasal discharge (4 scales with 1 = normal, moist and 4 = copious bilateral mucopurulent discharge) (Criteria, no date; University of Wisconsin-Madison, 2013;).

Body frame measurements (wither height, heart girth, hip height, hip width and body length) were recorded at arrival, week 4, 6, and 8 by the same person to ensure consistency of the measurements.

At the end of week 8, the calves were euthanized with a captive bolt followed by exsanguination. The entire gastrointestinal tract (GIT, from esophagus to rectum) was removed, weighed and dissected for sampling of corresponding GIT regions. Rumen tissues from cranial sac, ventral sac, and ventral blind sac and sections of jejunum were taken consistently from the same area and rinsed in 1×PBS to wash off digesta and fixed in 10% neutral buffered formalin solution for histomorphological analyses. Another section of jejunum was collected in ice-cold Dulbecco's modified Eagle medium and transported to lab immediately for analysis of paracellular and transcellular permeability using Ussing chambers. Jejunal mucosal samples were collected by gently removing the luminal surface by scraping off the tissue using a glass microscope slide, and then saved in liquid-N for extraction of RNA.

**Table 3.1. Nutrient composition of milk replacer and starter grain**

| Item | Milk replacer | Starter grain |
|---|---|---|
| DM, % | 93.4 | 85.9 |
| Crude protein, % of DM | 27.6 | 20.7 |
| Crude fat, % of DM | 14.4 | 2.8 |
| Ash, % of DM | 9.96 | 7.03 |
| $NE_M$, MJ/kg | 10.43 | 7.83 |
| $NE_G$, MJ/kg | 7.37 | 5.15 |
| Calcium, % of DM | 1.25 | 1.14 |
| Phosphorus, % of DM | 0.93 | 0.54 |
| Magnesium, % of DM | 0.14 | 0.43 |
| Potassium, % of DM | 2.34 | 1.27 |
| Sodium, % of DM | 0.80 | 0.28 |
| Iron, ppm | 64 | 152 |
| Manganese, ppm | 27 | 98 |
| Zinc, ppm | 47 | 169 |
| Copper, ppm | 4 | 41 |

### 3.3.3 Histomorphology

After fixation in 10% formalin solution for 3 d, the tissue samples for histomorphological analysis were dehydrated (Tissue-Tek VIP 5), embedded in paraffin block, sectioned (7 μm, 6-8 sections per tissue) and stained with Hematoxylin & Eosin Y. The whole slide was scanned for imaging using NanoZoomer digital pathology system (Hamamatsu Photonics, Japan). The morphology of rumen papilla (7 – 10 papillae per calf) were quantified for length, width and length-to-width ratio (L/W ratio), and jejunal villus (15 – 20 villi per calf) were quantified for villus height, crypt depth and villus height to crypt depth ration (V-to-C ratio).

### 3.3.4 RNA extraction and RT-qPCR

Total RNA was extracted from jejunal mucosa through phenol (TRIzol reagent)-chloroform method and subsequently diluted to 100 ng/μL. RNA purity was assess using NanoDrop 2000 spectrophotometers (ThermoFisher, MA). The ratios of 260/280 and 260/230 of all samples were above 1.8. Complementary DNA was reverse-transcribed from diluted RNA samples using

commercial kit (High capacity cDNA reverse transcription kit with RNase inhibitors, Applied Biosystems, MA). SYBR green was used as dye for real-time qPCR analysis (QuantStudio 3, ThermoFisher, MA). Relative mRNA expression of genes encoding occludin, claudin 1 and tight junction protein 1 was calculated through $2^{(\Delta\Delta Ct)}$ method.

### 3.3.5 Jejunum permeability

Gut tissue was opened along the mesenteric border and mounted in Ussing chambers (Physiologic Instruments, San Diego, CA) so that 0.5 $cm^2$ surface area of tissue was exposed to 2.5mL oxygenated Ringers with 10mM glucose on the serosal side and Ringers with 10mM mannitol on the luminal side at 37°C. The flux of FITC-4000 (FD-4; Sigma-Aldrich) was used to measure the paracellular pathway and the flux of horseradish peroxidase (HRP Type II; Sigma Aldrich) was used to measure the transcellular pathway. After 20-30 minutes of equilibration in the chambers, FD-4 was added at 400µg/mL and HRP at 200µg/mL to the mucosal compartment. Samples were collected from the serosal compartment at 30 minutes intervals. The concentration of FD-4 was measured using fluorescence at excitation 485 nm and emission 538 nm and HRP was measured using *O*-dianisidine substrate to detect HRP absorbance at 450nm.

### 3.3.6 Statistical analysis

All statistical analysis was performed in R (version 3.3.3) ) (R Core team, 2016). A random mixed model analysis was used to analyze data for body measurements, body weight, water intake, starter grain intake, milk replacer intake, rumen papillae length and width, jejunum villus length and crypt depth, gene expression and Intestinal permeability. The mixed model analysis was performed using the lme function in the nlme package (Pinheiro and Bates, 2017). A Multinomial logit model was used to analyze the data observed for health score. The multinomial logit analysis was performed using the multinom function in the nnet package (R Foundation for statistical computing, 2017a).

The model used for bodyweight, body frame, starter grain intake, water intake and milk replacer intake was a mixed model, where body weight at week 0 and bodyweight at week 0 squared was included as covariates:

$$y_{c,\,g,\,t} = \mu + \alpha_{g,\,t} + \beta_{c,\,g,\,t=0} + \beta^2{}_{c,\,g,\,t=0} + \gamma(\alpha)_{c,\,g,\,t} + \delta_c + e_{c,\,g,\,t}$$

where y = an observed value for body weight, body frame, starter grain intake, water intake or milk replacer intake. Where c is calf number (1-22), t is time (week, 1, 2, 3, 4, 5, 6, 7 or 8) and g is treatment group (C, L, H). µ indicates overall mean for the population, $\alpha_{g,\,t}$ is the fixed effect

of treatment group (g) at time (t). $\beta_{cgt=0}$ indicates start body weight of calf (c) in group (g) at time = 0 and $\beta^2_{c, g, t}$ is the start BW$^2$ of calf (c) in group (g) at time = 0. $\gamma(\alpha)_{c, g, t}$ is the random effect of calf (c) nested within group (g) at time (t), $\delta_c$ indicates fixed effect of calf breed and $e_{c, g, t}$ the error associated with the measurement taken from calf (c) from group (g) at time (t).

Pearson correlation (R Foundation for statistical computing, 2017b), using cor.test in R (version 3.3.3) ) (R Core team, 2016), between BW and wither height, hip height, hip width, body length and heart girth were tested using cor.test function in R. If correlation was non-significant this model was used:

$$y_{c, g, t} = \mu + \alpha_{c, g, t} + \beta_{c, g, t=0} + \beta^2_{c, g, t=0} + \rho_{c, g, t} + \gamma(\alpha)_{cg} + \delta_c + e_{c, g, t}$$

Where y is an observed value for bodyframe $\rho_{c, g, t}$ is the body weight for calf (c) in group (g) at time (t, week 4, 6 or 8). In the statistical analysis for bodyframe we had to take out one calf from the HIGH treatment group, due to a lot of health issues throughout the study

The model used for GIT weight, rumen pH, jejunum villie height and crypt depth and rumen papillae length and width was similar, but included bodyweight at week 8 as a covariate:

$$y_{c, g} = \mu + \alpha_{c, g} + \beta_{c, g, t=0} + \beta^2_{c, g, t=0} + \omega_{c, g, t=8} + \gamma(\alpha)_{c, g} + \delta_c + e_{c, g}$$

Where $\omega_{cg}$ indicates bodyweight at week 8 (t=8) of calf (c) in group (g). Rumen pH did not include bodyweight at week 0 and bodyweight squared at week 0. Rumen pH and GIT weight also included what order the calves were euthanized and what day out of two they were euthanized.

Jejunum permeability, included treatment and treatments interaction with time for measurements of flux. The model used for permeability was:

$$y_{c, g, t} = \mu + \alpha_{c, g} * \varphi_{c, g} + \gamma(\alpha)_{cg} + \delta_c + e_{c, g}$$

Where $\varphi_{c, g}$ is the time for measurement for calf (c) in group (g) at time (t).

The model used for gene expression in jejunum included no covariates:

$$y_{c, g, t} = \mu + \alpha_{g, t} + \gamma(\alpha)_{cg} + \delta_c + e_{c, g}$$

This model was used for all three genes investigated. For all the mixed model analysis stepAIC from the mass package was used to reduce the models (R Foundation for statistical computing, 2017c).

For health score a multinomial logit model was used, in order to analyze categorical data. This model accepts data as a matrix with various counts in the various categories. The model used was:

$$y_{c, g, t} = \mu + \theta_{c, t} + e_{c, g, t}$$

where y is the observed fecal score, nasal discharge score, respiration score or alertness score for the calf (c) in treatment group (g) at week (t). Here $\theta$ is the treatment for calf (c) at week (t).

For all the analysis that was performed in R significance was declared at $P < 0.05$ and a tendency of an effect was declared at $P < 0.10$. The R coding can be found in appendix B.

## 3.4 Results

### 3.4.1 Growth performance

Body weights week 1-8 were significantly ($P<0.05$) affected by the start body weight (body weight at week 0). In week 4 and 6 there was a tendency ($P<0.10$) for the LOW group to have a higher body weight than the HIGH group, LOW was numerically higher in body weight from week 2 until the end of the trial. Figure 3.1 shows the development in body weight over time.

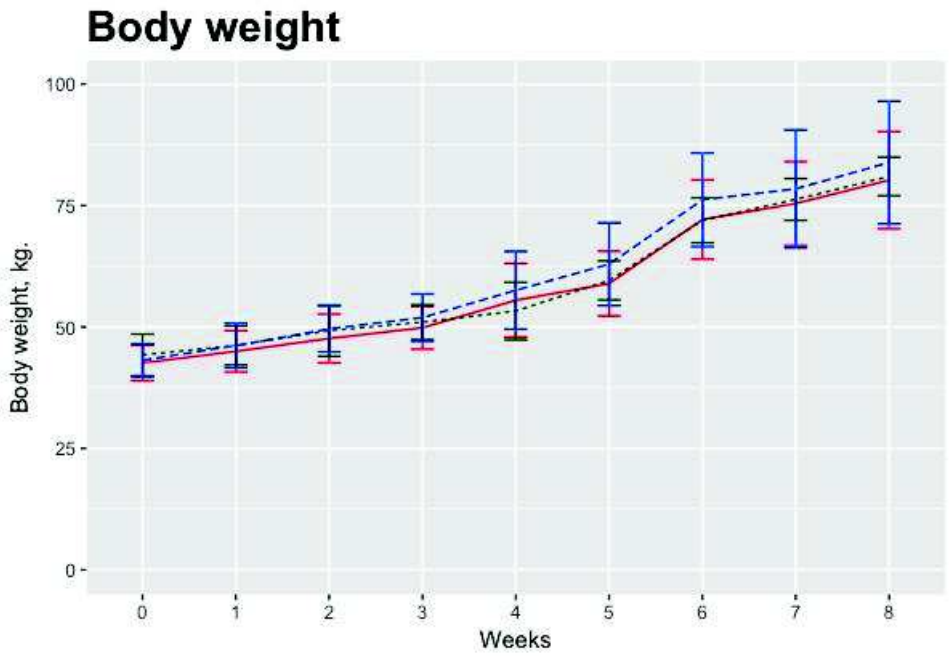



**Figure 3.1 weekly mean body weight for CON, LOW and HIGH, Red line is CON, Blue dashed line is LOW and green dotted line is HIGH.**

Body frame measurements at the time of euthanizing, week 8, is shown in table 3.2 Body length and heart girth were significantly ($P<0.05$) positively correlated with bodyweight, Hip width and wither height had a tendency ($P<0.10$) of a positive correlation with bodyweight and Hip height were not ($P>0.10$) correlated with bodyweight. In week 4 start body weight affected Body length

and hip width, in week 6 start body weight affected heart girth and in week 8 start body weight affected hip width, hip height and wither height. In week 4 HIGH had a significantly (P<0.05) higher body length than CON. In week 6 LOW tended (P<0.10) to have a higher heart girth than CON, HIGH tended (0.10) to have a higher hip height than CON and LOW had a significantly (0.05) higher hip width than HIGH and CON. In week 8 LOW had a significantly (P<0.05) higher wither height than CON, and the wither height tended (P<0.10) to be higher in LOW compared to HIGH as well.

**Table 3.2 Body weight and Body frame measurements at time of euthanizing the calves**

| | CON | SD | LOW | SD | HIGH | SD | P-value |
|---|---|---|---|---|---|---|---|
| n | 8 | | 7 | | 7 | | |
| Final BW | 80.21 | 10.0 | 83.86 | 12.62 | 81.00 | 3.96 | NS |
| **Body frame** | | | | | | | |
| Body length | 86.43 | 1.72 | 88.67 | 3.44 | 87.17 | 2.64 | NS |
| Heart girth | 100.71 | 4.61 | 102.14 | 4.49 | 100.08 | 3.12 | NS |
| Wither height | 86.36[a] | 3.00 | 90.35[b] | 2.71 | 87.99[a] | 2.44 | 0.003 |
| Hip height | 91.53 | 4.07 | 94.25 | 2.74 | 93.40 | 2.17 | 0.04 |
| Hip width | 22.13 | 1.24 | 22.95 | 1.53 | 22.12 | 0.74 | NS |

[a, b] *Same row not bearing a common subscript differ (P≤0.05)*

### 3.4.2 Milk replacer -, grain – and water intake

Milk replacer intake was significantly affected by start body weight, like body weight was, from week 2-8. LOW had a significantly (P<0.05) increased intake of MR for week 5 and 7, when compared to HIGH. no significant difference was observed between CON and HIGH or CON and LOW. This tendency (P<0.10) was also seen in week 4. In figure 3.2 milk replacer intake is shown.

Starter grain intake was not affected negatively by butyrate supplementation. In the fourth week of the trial, LOW had I significantly (P<0.05) increased starter grain intake compared to HIGH. Starter grain intake is shown in figure 3.3.

Water intake tended to be affected by supplementing butyrate, in week 2 and 4 a tendency (P<0.10) of the LOW group having an increased water intake compared to HIGH was seen. Figure 3.4 shows the water intake.

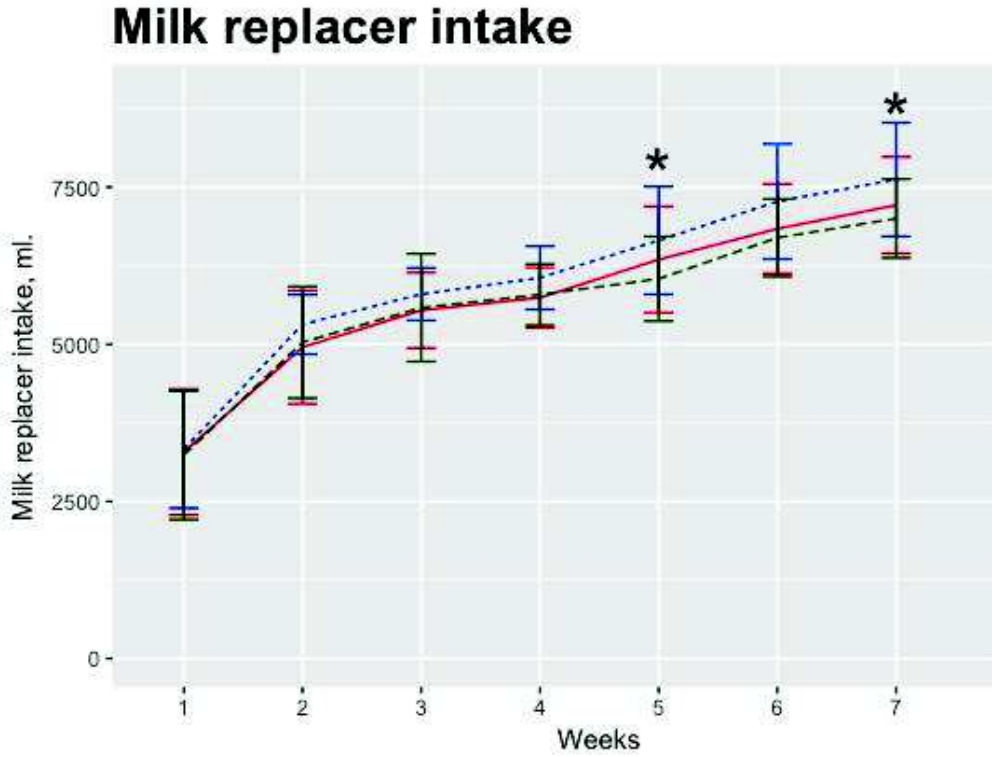



**Figure 3.2 Weekly milk replacer intake of CON, LOW and HIGH, LOW and HIGH, Red line is CON, Blue dotted line is LOW and green dashed line is HIGH, stars indicate significant (P<0.05) differences between groups.**

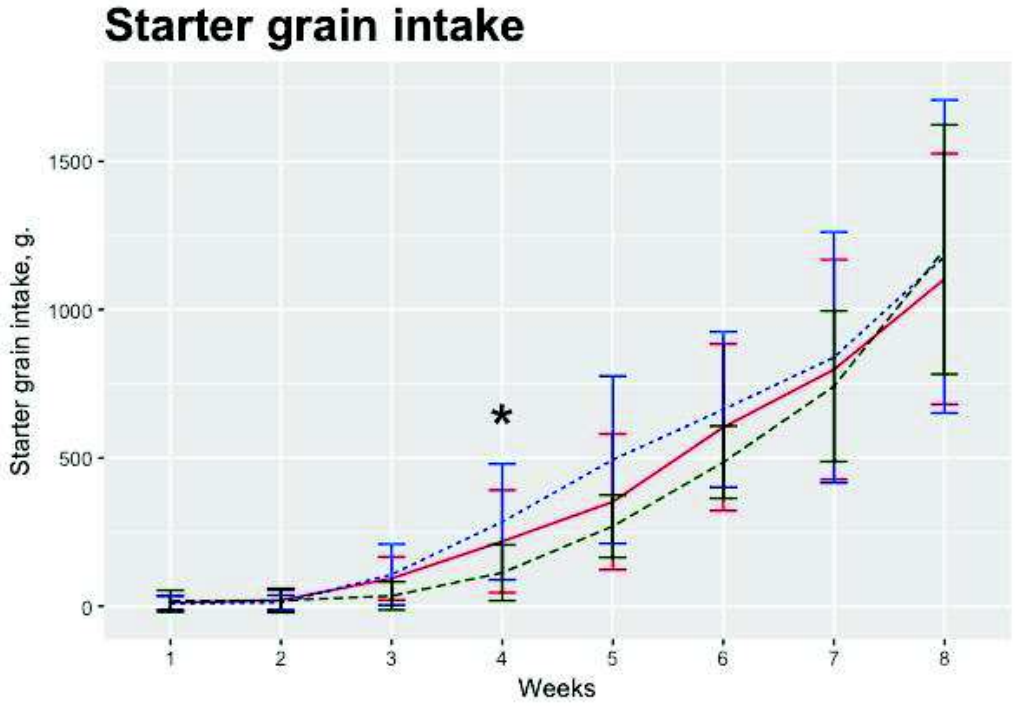



**Figure 3.3 Weekly starter grain intake of CON, LOW and HIGH, Red line is CON, Blue dotted line is LOW and green dashed line is HIGH, stars indicate significant (P<0.05) differences between groups.**

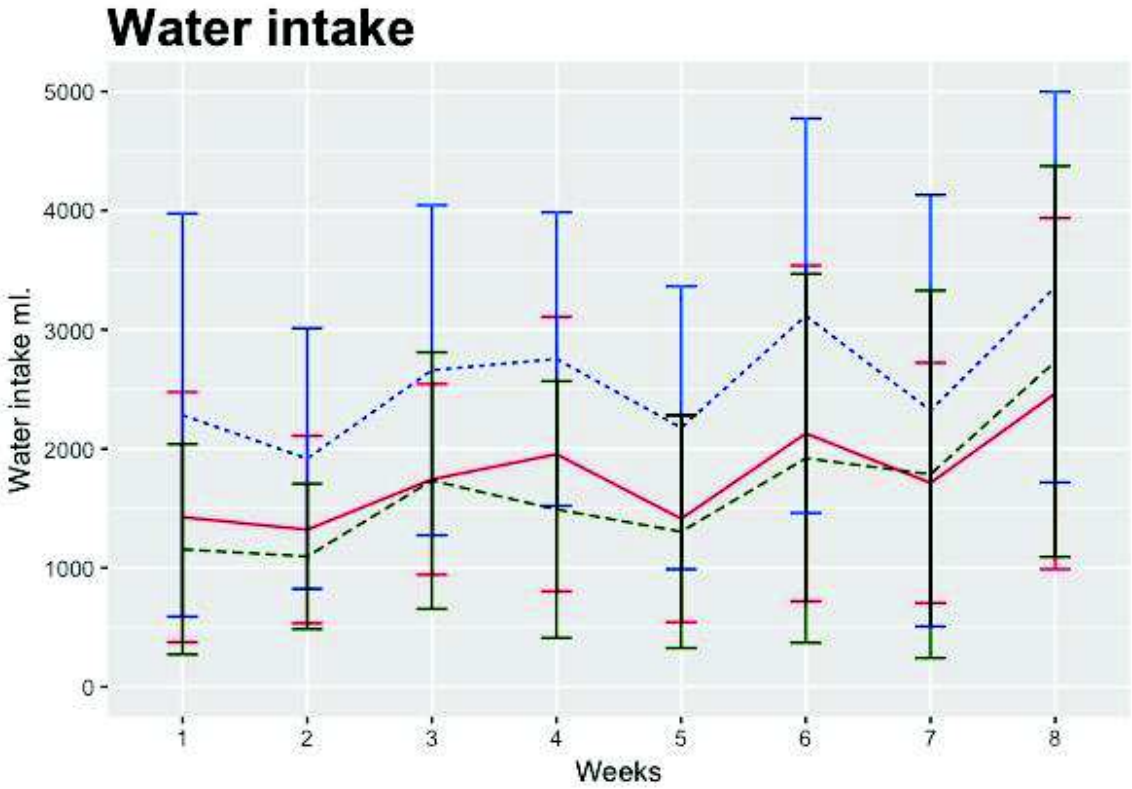



**Figure 3.4 Weekly water intake of CON, LOW and HIGH, Red line is CON, Blue dotted line is LOW and green dashed line is HIGH.**

### 3.4.3 Health Performance

Frequencies for health scores are shown in table 3.3. As we can see in table 3.3 for nasal discharge score, respiratory score and alertness, only few calves had a score over 1. This means butyrate supplementation did not affect nasal discharge, respiration and alertness. When looking at fecal score, more calves had a score over 2. The multinomial logit analysis showed that fecal score was not worsened by supplementation with butyrate.

**Table 3.3. Health score frequencies, percent calves/days with the given score in the given week in the given treatment**

| | Score | Week 1 | | | Week 2 | | | Week 3 | | | Week 4 | | | Week 5 | | | Week 6 | | | Week 7 | | | Week 8 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CON | LOW | HIGH | CON | LOW | HIGH | CON | LOW | HIGH | CON | LOW | HIGH | CON | LOW | HIGH | CON | LOW | HIGH | CON | LOW | HIGH | CON | LOW | HIGH |
| Alertness | 1 | 98% | 100% | 100% | 98% | 98% | 92% | 98% | 100% | 98% | 100% | 100% | 100% | 100% | 100% | 90% | 95% | 97% | 100% | 100% | 86% | 100% | 100% | 100% | 100% |
| | 2 | 2% | | | 2% | 2% | 4% | | | 2% | | | | | | 10% | 5% | | | | 11% | | | | |
| | 3 | | | | | | 2% | | | | | | | | | | | 3% | | | 3% | | | | |
| | 4 | | | | | | 2% | 2% | | | | | | | | | | | | | | | | | |
| | 5 | | | | | | | | | | | | | | | | | | | | | | | | |
| Fecal | 1 | 79% | 78% | 78% | 33% | 38% | 21% | 81% | 87% | 83% | 88% | 91% | 94% | 87% | 83% | 90% | 93% | 92% | 94% | 92% | 100% | 84% | 89% | 89% | 72% |
| | 2 | 10% | 11% | 7% | 38% | 38% | 31% | 15% | 8% | 12% | 12% | 9% | 6% | 9% | 11% | 8% | 5% | 8% | 6% | 5% | | 11% | 11% | 7% | 24% |
| | 3 | 8% | 7% | 7% | 24% | 19% | 27% | 4% | 5% | 2% | | | | 2% | 6% | 2% | 2% | | | 3% | | 5% | | 4% | 3% |
| | 4 | 4% | 4% | 9% | 5% | 6% | 21% | | | 2% | | | | 2% | | | | | | | | | | | |
| | 5 | | | | | | | | | | | | | | | | | | | | | | | | |
| Respiration | 1 | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 98% | 100% | 100% | 91% | 100% | 85% | 100% | 100% | 94% | 100% | 97% | 95% | 96% | 100% | 93% |
| | 2 | | | | | | | | | | 2% | | | 7% | | 8% | | | 6% | | | 5% | 4% | | 3% |
| | 3 | | | | | | | | | | | | | 2% | | 6% | | | | | 3% | | | | 3% |
| | 4 | | | | | | | | | | | | | | | | | | | | | | | | |
| | 5 | | | | | | | | | | | | | | | | | | | | | | | | |
| Nasal discharge | 1 | 100% | 100% | 100% | 100% | 98% | 98% | 98% | 100% | 100% | 93% | 89% | 97% | 95% | 85% | 96% | 100% | 89% | 100% | 97% | 100% | 97% | 100% | 96% | 100% |
| | 2 | | | | | 2% | 2% | 2% | | | 5% | 6% | 3% | 4% | 6% | 4% | | 5% | | | | 3% | | 4% | |
| | 3 | | | | | | | | | | 2% | 3% | | 2% | 6% | 2% | | 3% | | 3% | | | | | |
| | 4 | | | | | | | | | | | | | | 2% | | | 3% | | | | | | | |

### 3.4.7 Papillae morphology, rumen pH and gastro intestinal tract weight

GIT weight, rumen pH, rumen papillae length, rumen papillae width and length to width ratio is shown in table 3.4. There was a significant effect of start body weight on the GIT weight. pH was significantly affected by body weight at euthanizing, but was not affected by treatment. Papillae length and papillae length to width ratio was affected by start bodyweight and bodyweight at week 8. Papillae length tended ($P<0.10$) to be affected by treatment, CON had significantly longer papillae than LOW. Papillae length to width ratio was significantly ($P<0.05$) affected by treatment, CON had a significantly higher ratio than LOW. Papillae width was not different between groups.

**Table 3.4 GIT weight, Rumen pH, jejunum morphology and rumen morphology**

| | CON | SD | LOW | SD | HIGH | SD | P-value |
|---|---|---|---|---|---|---|---|
| GIT weight | 13.46 | 0.97 | 13.42 | 3.08 | 13.64 | 1.93 | NS |
| Rumen pH | 6.43 | 0.85 | 6.27 | 0.87 | 6.48 | 0.66 | NS |
| **Rumen, Cranial sac** | | | | | | | |
| Papillae length | 2363.53[a] | 754.55 | 2020.93[a] | 768.36 | 2075.39[a, b] | 781.99 | 0.07 |
| Papillae width | 456.16 | 188.08 | 492.62 | 144.23 | 499.06 | 198.21 | NS |
| L-to-W ratio | 5.60[a] | 2.01 | 4.30[b] | 1.81 | 4.47[a, b] | 1.77 | 0.02 |
| **Middle jejunum** | | | | | | | |
| Villus height | 524.16 | 291.81 | 726.42 | 272.42 | 598.34 | 166.20 | NS |
| Crypt depth | 273.66 | 142.19 | 281.84 | 135.32 | 297.15 | 140.98 | NS |
| V-to-C ratio | 2.25[a] | 1.30 | 3.08[b] | 1.54 | 2.48[a, b] | 1.30 | 0.06 |
| **Middle jejunum, permeability** | | | | | | | |
| n | 5 | | 5 | | 4 | | |
| HRP flux | 47.83 | 55.90 | 40.25 | 36.89 | 83.12 | 85.80 | NS |
| FD4 flux | 445.13 | 420.35 | 394.66 | 356.82 | 700.68 | 407.68 | NS |

[a, b] *Same row not bearing a common subscript differ ($P \leq 0.05$)*

### 3.4.4 Jejunum morphology

In table 3.4 villus height, crypt depth and villus height to crypt depth ratio mean and SD for the groups are shown. There was no effect of treatment on villus height and crypt

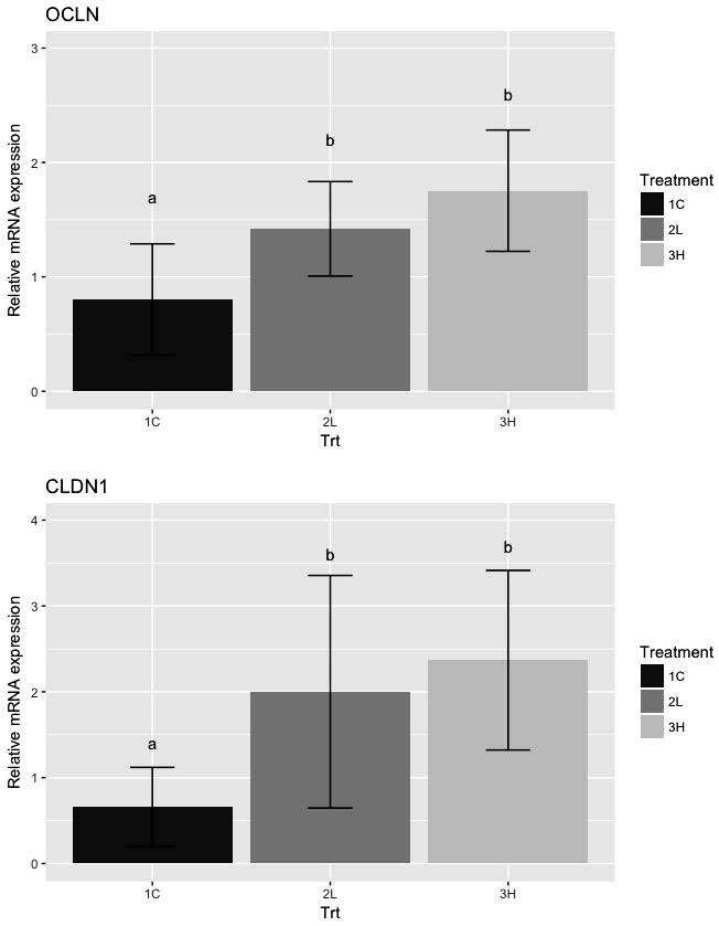
depth. As shown in table 3.4 there was a tendency (P<0.10) of a treatment effect on villus height to crypt depth ratio. LOW had a significantly (P<0.05) higher ratio compared with CON.

### 3.4.5 Jejunum permeability

In table 3.4 jejunum permeability, FD4 flux and HRP flux, mean and SD for each group are shown. No significant differences between the groups were found in FD4 flux or in HRP flux. High SD was found for all three treatment groups, for CON and LOW SD was almost the same as the mean flux for the treatment group.

### 3.4.6 Jejunum relative mRNA expression

Figure 3.5 shows the relative mRNA expression of occluding (OCLN), claudin 1 (CLDN1) and Tight junction protein 1 (TJP1). For CLDN1 and OCLN it was seen that LOW and HIGH were significantly (P<0.05) higher in relative mRNA expression compared to CON. There were no significant differences between groups when looking at TJP1.



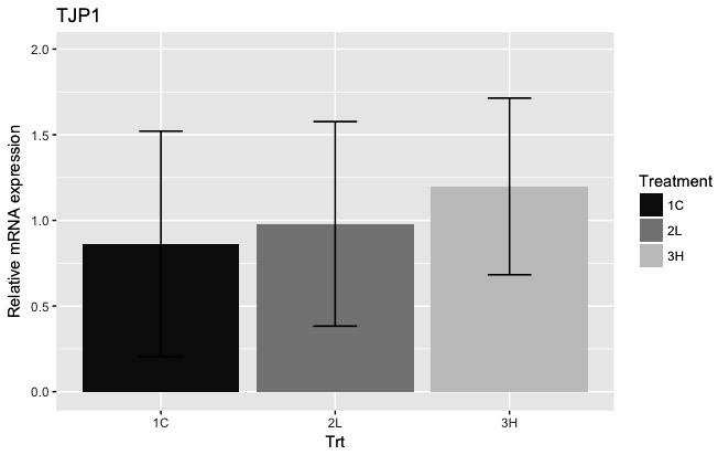



**Figure 3.5 Relative mRNA expression of occludin (OCLN), claudin 1 (CLDN1) and tight junction protein 1 (TJP1)**

## 3.5 Discussion

This trial varies from other trials in the type of butyrate supplemented, to our knowledge this is the first trial using monobutyrin as supplementation in milk replacer. In this trial, there was a LOW and a HIGH group, LOW seems to have better performance and GIT development compared to HIGH.

### 3.5.1 Calf growth

Even though we didn't see a significant effect on body weight, it was found that calves in the LOW treatment group had a better growth than CON and HIGH. This was seen by a tendency ($P<0.10$) of a higher body weight at week 4 and 7, it was also seen numerically from week 2 and on to week 8 at slaughter, and by a significantly ($P<0.05$) higher wither height and hip height for LOW compared to CON at slaughter this was also seen numerically in body length, heart girth and hip width. This increased growth could be due to the significantly and numerically higher milk replacer intake and starter grain in LOW calves compared to CON and HIGH. Thus, butyrate in the form of monobutyrin increased growth of the supplemented calves is in agreement with findings on increased ADG for Na-butyrate or calcium coated butyrate supplemented calves in studies by Guilloteau *et al.* (2009b), Nazari *et al.* (2012), Kato *et al.* (2011), Górka *et al.* (2011) and Górka *et al.* (2009).

The lack of growth response to monobutyrin supplementation in HIGH treatment group compared to LOW treatment group, could be because the HIGH treatment group has the lowest numerical MR intake week 5 - 7, starter grain intake week 3 - 7 and water intake week 1 - 6 compared to LOW and CON. This might be because the higher dose of monobutyrin gives a

different taste or due to the secretion of leptin. It has been seen in vitro that increasing amounts of butyrate increases the secretion of leptin, which can lead to a decrease in feed consumption in the calf (Yonekura et al., 2003), though this has not been studied extensively in vivo yet. Even though HIGH calves had the lowest feed intake, they still attained the same body weight curve and bodyframe measurements as CON calves. It was hypothesized that a low supplementation of monobutyrin fed to preweaned calves in MR, would have a positive effect on calf growth. The LOW treatment group did seem to have a higher growth, expressed as body weight and body frame along with a higher feed intake.

### 3.5.2 Rumen development

Rumen development was not positively affected by monobutyrin supplementation in contrast to what was hypothesized. Rumen papillae length and rumen papillae length to width ratio in the cranial sac was decreased in LOW and HIGH treatment groups compared to CON. These findings are in contrast to Gorka *et al.* (2009) and Górka *et al.* (2011), who found that supplementing Na-butyrate had a positive effect on rumen papillae length in the cranial ventral sac and cranial dorsal sac. These contradictory results might be due to older calves, calves in those other studies compared to the present study where calves were approximately 56 days at slaughter, the calves in the studies by Gorka *et al,* (2009) and Górka *et al.* (2011) were 26 days of age. It might be because in this study we used monobutyrin, a form of butyrate that passes through the forestomach's and thereby doesn't start getting digested before it enters the small intestine, where as Gorka *et al.* (2009) and Górka *et al.* (2011) used Na-butyrate. Rumen cranial sac papillae width were numerically higher in HIGH and LOW, compensating for the shorter papillae, this gives a bigger surface for absorption in the rumen. These results is in agreement with findings by Gorka *et al.* (2009) and Górka *et al.* (2011). It was hypothesized that a low supplementation of monobutyrin fed to preweaned calves in MR, would enhance rumen development. It was seen that supplementation seemed to decrease papillae length and papillae length to width ratio, whereas it did increase papillae width, compensating for the length.

### 3.5.3 Jejunum development

In mid jejunum, a higher villus height to crypt depth ratio was found in the LOW treatment group compared to CON and HIGH, due to an increased villus length in LOW compared to CON and HIGH. These results are contradictory with results found by Guilloteau *et al.* (2009b) and Górka *et al.* (2014) they found shorter villi in Na-supplemented calves. These

contradictory results might in part be due to differences in slaughter age which were 151 days of age and 26 days of age in the previous study compared to approximately 56 days of age in the present. The increased villus length and villus length to crypt depth ratio supports our hypothesis that monobutyrin in the small intestine increases absorptive capacity, thereby an improving utilization of the feed, which can lead to an improved growth in calves on the LOW treatment.

As mentioned before HIGH was able to maintain the same growth as CON even though HIGH had a lower feed consumption, this could also partly be because of higher papillae width and partly due to higher tight junction protein relative mRNA expression in HIGH compared to CON. LOW and HIGH did show a higher relative mRNA expression in genes encoding occludin and claudin-1, this is consistent with the better histological development found in villus height and villus height to crypt depth ratio. The fact that this increased development of the intestine, in HIGH was almost as high as LOW, could indicate that the reason for HIGH to not have the same increased growth as low could be because of the decreased feed intake. It was hypothesized that a low supplementation of monobutyrin fed to preweaned calves in MR, would enhance small intestine epithelial development. It was found that villus length, villus length to crypt depth ratio and tight junction protein relative mRNA expression was enhanced in LOW treatment group

### 3.5.4 Health

The calves in this study were generally healthy, the frequency of diarrhea in the calves were the same across treatment groups, even though relative mRNA expression of tight junction proteins were higher in LOW and HIGH calves. A difference in the frequency of scours and days of electrolyte was seen in other studies though, in studies by Górka *et al.* (2011) and Górka *et al.* (2009) days with scours and days on electrolytes were lower in calves supplemented with Na-butyrate in starter grain or MR, respectively. It was hypothesized that a low supplementation of monobutyrin fed to preweaned calves in MR, would positively affect calf health. It was found that supplementation did not have a negative effect, but due to generally healthy calves it did not have a more positive effect than in the CON treatment group either.

### 3.5.5 Calf performance

The positive effect on intestinal development in LOW treatment group might contribute to a higher digestibility in the small intestine end thereby a higher rate of passage. This increased passage rate may partly explain the surprisingly higher feed intake of MR and textured starter grain in LOW treatment group compared to CON. The increased feed

intake in LOW also gives a higher water intake in LOW, also contributing to the increased digestibility and passage rate. Another factor that could explain the difference in feed intake between LOW and HIGH could be appetite regulating hormones like leptin. It has been found that butyrate may have an effect on appetite when supplementing butyrate, increasing butyrate supplementation increases the secretion of leptin. Leptin is signaling the brain that the animal is full, and the intake of that animal will be decreased (Guilloteau et al., 2010). Several factors could play a role in regulating feed intake in butyrate supplemented calves. For further studies, it would be interesting to look into some of the factors, as Leptin, passage rate and digestibility in butyrate supplemented calves.

## 3.6 Conclusion

Based on these results it can be concluded that a low dose of monobutyrin supplementation in milk replacer in a low dose 0.37% of DM tended to positively affect the growth of calves, this might be due to the positive effect on milk and starter intake, potentially due to increased digestive and absorptive function due to improved jejunal villi development, and epithelial integrity through higher mRNA expression of claudin 1 and occludin. However, further studies are needed on the effect of supplementing milk replacer with monobutyrin to confirm these results and to determine the effect on the long-term consequences for rumen epithelial development.

# 4. Overall discussion

The overall hypothesis for the present project were that ingested nutrients, more specific the SCFA butyrate, has a stimulatory effect on calf growth, calf health, small intestinal development and rumen development. In order to investigate this hypothesis, the development of the calves GIT was investigated and nutrients like protein, carbohydrates and fatty acids and their effect on GIT function and development was investigated through a literature study. The effect of supplementing Na-butyrate and calcium coated butyrate was investigated through a literature study and the effect of supplementing monobutyrin were researched in the research study included in this thesis.

## 4.1 Calf feeding and effects on calf growth, health, rumen- and small intestinal development

When comparing MR to whole milk, it was found that calves fed whole milk had a higher growth, seen as a higher ADG and weaning weight compared to calves fed MR (Bar-Peled et al., 1997; Moallem et al., 2010; Shamay et al., 2005). Feeding the calf whole milk means the farmer has to use milk from the cows that could be a source of income to feed the calves. It was discovered that this higher growth as a calf did not affect the calf's milk production capacity as a cow negatively, it results in the same milk yield as when no accelerated growth is seen, and may even result in higher milk yield. If MR supplemented with butyrate could give as high a growth as whole milk, and have a similar positive effect on milk yield, without too high a price on the MR, this might be a better solution on how to feed calves for the farmer. But how much butyrate and how many times a day? In the literature review it was concluded that supplementing 3 g. of sodium butyrate in the milk replacer fed 2 times a day has been seen to have a positive effect on rumen development and calf growth. In the following, results from the literature review versus the result from the present study will be discussed, in the end a recommendation for feeding calves will be concluded. In the literature review, it was found that the calves GIT is designed for digesting carbohydrate and protein from milk. Therefore, protein and carbohydrate in MR where the same as found in milk.

Monobutyrin is a monoglyceride of butyric acid. Monobutyrin has been found to have almost as good antimicrobial activities as butyric acid. *Salmonella* where inhibited best by butyric acid followed by monobutyrin and *clostridium perfringens* where attenuated by both butyric acid and monobutyrin (Bedford and Gong, 2017). When butyrate is fed as monobutyrin,

butyrate needs to be released from glycerol before it can elicit an effect on GIT. Some of the glycerol is expected to be cleaved in the abomasum due to the pregastric lipase activity, however most of the monobutyrin is expected to reach the proximal part of the small intestine (Górka et al., 2018). This form of butyrate was chosen because of the ability to not be digested before reaching the small intestine and because of its antimicrobial activities. Na-butyrate has been researched before, and shows to have an effect on rumen development but shows no significantly stimulating effect on small intestine. In the present study, we wanted to study butyrates effect on small intestine, and to see if digestion and absorption in the small intestine could have a feedback effect, stimulating rumen development. Therefore, we chose to use monobutyrin and not Na-butyrate as previously studied.

When supplementing monobutyrin versus Na-butyrate, monobutyrin escapes the forestomach's and is not digested before it reaches the small intestine, explaining the limited effect found on rumen development in the present study. Whereas Na-butyrate may get digested already in the forestomach's or abomasum of the calf, which means Na-butyrate would not reach the small intestines, but would be digested in the forestomachs. A blend of monobutyrin and Na-butyrate might be the basis for more research. In the literature review we found that calf growth was significantly positively affected by Na-butyrate or calcium coated butyrate supplementation this was seen as a higher ADG and BCS. These studies either had the same number of calves in the treatment groups (n=7 or 8) or more calves (n= 12, 17 or 44) than the present study (LOW & HIGH n=7, CON n=8) (Gorka et al., 2009; Górka et al., 2014, 2011a; Guilloteau et al., 2009b; Kato et al., 2011; Nazari et al., 2012). Monobutyrin seem to have the same increasing effect on body weight and body frame, as Na-butyrate or calcium coated butyrate, though in the present study body weight and body frame differences were mostly seen as a tendency or numerically. Supplementing Na-butyrate also positively affected rumen development, through increased growth of rumen papillae length and width and a larger rumen as % of whole stomach weight (Gorka et al., 2009; Górka et al., 2011a; Kato et al., 2011). In the present study these results, showing a positive effect on rumen development, were not replicated. Papillae length and length to width ration were significantly higher in CON compared to LOW, Papillae width were numerically highest in HIGH, then LOW compared to CON. It is not clear why the results on rumen development weren't the same, but the reason could be the form of butyrate supplemented. Using monobutyrin, which largely escapes digestion in the forestomachs, might not stimulate the same rumen development as using Na-butyrate. Na-butyrate seemed to have a relatively small effect on small intestinal development (Górka et al., 2014; Guilloteau et al., 2009b). Whereas monobutyrin significantly increased villus height to crypt depth ratio through a

numerically increased villus height in LOW compared to CON and HIGH. This difference might also be due to the absorption site of Na-butyrate and monobutyrin. Monobutyrin is mainly absorbed in the intestines whereas Na-butyrate can be absorbed in the forestomachs. The relatively small effect on small intestinal development from supplementing butyrate in milk replacer might in Górka *et al.* (2014) and the present study be due to the small treatment groups (n=7 or 8), and in Guilloteau *et al.* (2009) be due to the older calves (151 days of age), in Guilloteau *et al.* (2009) the calves has started eating more grain, affecting rumen and small intestinal development, that might be the reason for a small effect on intestinal development. This positive effect on small intestinal development was also seen through an increase in some of the tight junction proteins in the present study. This could lead to an increase in the absorption of nutrients and digestion in the small intestine. In the present study, LOW treatment group had an increased growth and better small intestinal development, found by seeing longer villus and higher villus length to crypt depth ratio. This suggest that the low dose (0.37% of DM) of monobutyrin has the best effect. The promoted growth and intestinal development when feeding the low dose might be due to the higher starter grain intake in the present study, higher starter grain intake and wat intake could increase digestibility and passage rate of the feed in the calf. This improved intestinal integrity, might be the explanation to why improved health, in specific less diarrhea, which is a common disease in young calves, is seen in calves supplemented with butyrate (Gorka et al., 2009; Górka et al., 2011a). In the literature review a dose of approximately 0.3% of DM or as fed per day in the MR fed 2 times a day, with ad libitum access to starter grain seemed to be optimal for an increased calf growth, calf health and promoting rumen development. In the present study 0.37% of DM fed 2 times a day, with ad libitum access to starter grain, seemed to be optimal for an increased calf growth, keeping calves healthy and promoting intestinal development.

## 4.2 Long-term productivity

The accelerated growth that is seen when supplementing calves with butyrate in the MR, might have long-term consequences on growth and milk yield. In the literature review it was found that when accelerated growth in calves are seen, it may not have a negative effect on future milk production, it may even give a higher milk yield. These results were seen for accelerated growth until weaning, after weaning calves were fed the same and had similar growth, until calving (Bar-Peled et al., 1997; Moallem et al., 2010; Morrison et al., 2009; Shamay et al., 2005; Terré et al., 2009). When looking at literature results for weaning weight, studies where long-term effects of feeding MR versus whole milk is investigated, whole milk fed

calves has higher weaning weight. When comparing the weaning weight of the calves fed whole milk in these studies, taking weaning age into consideration as well, calves supplemented with butyrate seem to have just as high a body weight as calves fed whole milk. This means that MR supplemented with butyrate may give the same positive effect on calf growth as feeding whole milk. This means that the farmer will have the same effect on growth in his calves using MR. Which means the farmer would be able to use all of his produced milk to sell and produce cows, that might have either the same or higher milk yield as cows fed MR without butyrate as calves or same effect on milk yield as cows fed whole milk as calves.

### 4.3 How should the preweaning calf be fed?

From this literature study and research project it seems clear that due to the undeveloped forestomachs, it is limited what type of feedstuffs and nutrients the young preruminant calf can digest. The GIT in the calf is designed to digest nutrients naturally present in cow milk. Fatty acids are naturally present in cow's milk, the SCFA, butyrate, is also naturally present. It seems clear from this study that supplementing Na-butyrate, calcium coated butyrate and monobutyrin has a positive effect on the calf, increasing calf growth rate and promoting calf health. Although monobutyrin did not have a positive effect on rumen development, Na-butyrate did. When looking at small intestinal development, only few significantly positive results were found for Na-butyrate. Monobutyrin might not be optimal for stimulating rumen development, therefore a blend of monobutyrin and Na-butyrate might be optimal for stimulating rumen and small intestinal development. From this study, it would be recommended to use MR supplemented with a blend of monobutyrin and Na-butyrate. This conclusion was drawn since monobutyrin seemed to have a slightly negative effect on rumen development, but a positive effect on small intestinal development. Na-butyrate seemed to have a positive effect on rumen development but only a small effect on small intestinal development. The optimal dose seems to be approximately 0.3% of DM or as fed per day or 0.37% of DM, if increasing the amount to 0.75% of DM calf growth may be similar to calves not supplemented, meaning calf growth may not be stimulated. The MR should be fed twice a day and the calf should have access to ad libitum calf starter.

## 5. Conclusion and perspectives

Based on the results found in the literature and in this research trial Na-butyrate and monobutyrin seems to have a positive effect on calf growth and health, leading to less

diarrhea in preruminant calves when supplemented in MR, calves were generally healthy throughout the present study. Rumen development Has been found in other studies to be positively affected by supplementation of Na-butyrate, increasing rumen size and papillae length and width, but this does not seem to be the case when supplementing monobutyrin, which decreased papillae length and length to width ratio, but monobutyrin seemed to stimulate small intestinal development and small intestinal barrier function. Results on intestinal development is contradictory, most results are seen numerically and in literature it was found that in mid jejunum villus height and crypt depth were decreased in Na-butyrate supplemented calves whereas in the present study it was found that monobutyrin had tendency of increasing villus height to crypt depth ration and numerically increase villus height and crypt depth. It can be concluded that the relative mRNA expression of tight junction proteins is positively affected by monobutyrin supplementation and possibly absorptive capacity and thereby possibly increasing intestinal integrity, promoting less diarrhea. More research is needed to study the effect of monobutyrin in combination with Na-butyrate supplementation on rumen development and small intestinal development in preruminant calves supplemented in MR. A study where both monobutyrin and Na-butyrate is supplemented, possibly as a blend in MR or monobutyrin in the milk and Na-butyrate in the calf starter grain, since monobutyrin is digested in the small intestines and Na-butyrate can be digested in the rumen. Thereby a positive effect on both rumen and small intestinal development should be seen.

Further studies on the effect on intestinal development of calves is needed and studies on the long-term effects of supplementing butyrate would be relevant. It has been investigated what effect accelerated growth by feeding whole milk instead of milk replacer has, but it has not been studied when supplementing butyrate. It would be relevant too look at if the improved rumen development, could promote an earlier age for weaning, meaning calves would consume more grain at an earlier age. This increased intake could promote higher ADG and faster body frame growth. This accelerated growth could lead to earlier puberty and earlier insemination and thereby earlier calving, which means the calf/cow would start “paying for itself” in an earlier age by milk production. It has been seen that the milk yield of the cow is positively correlated with the body weight, this means that as long as the cow weights at calving the same or more as a cow that wasn’t supplemented with butyrate as a calf, milk yield would not be negatively affected.

Further studies on the amount of butyrate supplemented would also be relevant. The articles reviewed in this thesis, were they supplement calves with sodium butyrate, in 6 out of 7 of the studies the calves were supplemented with 0.3% of DM or 3 g./day, one study used a stepwise increase in butyrate but that seemed to have a negative effect. So, further research using

different amounts, but a steady amount through the study. It has been seen that butyrate in high amounts can increase leptin production, which decreases appetite and thereby the feed intake will be decreased. It would therefore be relevant to find the amount with maximum positive effect on calf growth, rumen and intestinal development without affecting feed intake negatively would be a relevant research area. Research has been made on supplementing in starter grain, and few trial has been done on supplementing in both starter grain and milk replacer with Na-butyrate. Researching the right combination of amounts and blends of Na-butyrate and monobutyrin in grain starter and milk replacer to reach optimum growth and GIT development would be interesting. This would also be a way of studying the long-term effects, supplementing in starter grain would possibly lead to a longer period of accelerated growth, it would be interesting to see this effect on age when reaching puberty, calving age and milk yield.

Implementing MR supplemented with butyrate out on the farms requires that there is no economic loss for the farmer but an economic gain. It is commonly used to feed the calf approximately 6 - 8 liters of milk the first 60 days after birth. When calculating varying the milk price and using a standard price of 12 danish kroner/kg MR, the price on milk needs to be around 2,30 danish kroner/kg milk before the farmer has an economic gain from feeding MR. Using MR instead of whole milk on the farms, does not give an economic gain before the milk price is at least 2,30 danish kroner/kg milk. This is calculated assuming that the MR is supplemented with butyrate, leading to accelerated growth, giving the same or a higher calf growth and future milk yield, as feeding whole milk to the calf. In order for implementing MR supplemented with butyrate on the farms it needs to be further investigated what form of butyrate or blend of monobutyrin and Na-butyrate gives the highest calf growth, calf health, rumen development and small intestinal development. In these investigations, the long-term effect on future milk yield should also be included and the milk price needs to be at least 2,30 danish crowns/kg milk or the supplemented MR needs to be able to increase future milk yield in supplemented calves.

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Žitňan, R., Voigt, J., Wegner, J., Breves, G., Schröder, B., Winckler, C., Levkut, M., Kokardová, M., Schönhusen, U., Kuhla, S., Hagemeister, H., Sommer, A., 1999. Morphological and functional development of the rumen in the calf: Influence of the time of weaning 1. Morphological Development of Rumen Mucosa. Arch. Anim. Nutr. 52, 351–362.

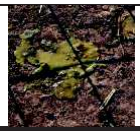
# Appendix A

## Health scoring card

### J1602_Calf Health Check and Scoring Chart

**Health checking instruction:**

1. **This is task No.1 for each shift. Bring chalk, flashlight and datasheet with you.**
2. **Avoid direct contact with calf, but stimulate them to stand up if any score > 2.**
3. **Mark "X" on the back wall of the hutch, if the calf has any score > 2.**
4. **Mark "√" on the back wall of the hutch, if there was "X" previously and the score become < or = 2 during your health checking.**
5. **Make the same note on your datasheet and the whiteboard near water station.**
6. **If there were 4 sign of "√" in a row, please erase all the signs on the back wall.**

| Score | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **Appearance** | Alert, bright-clear eyes, ears up | Slightly slow response, slightly droopy ears | Lethargic, head & ears droop, eyes dull or sunken, | Unable to stand, head & ears droop, eyes dull or sunken, | flat on side, Severe depression |
| |  |  |  | | |
| **Fecal Score** | Firm, well formed (not hard) | Soft, pudding-like | Runny, pancake batter | Liquid, splatters | |
| |  |  |  |  | |
| **Respiratory Score** | Normal breath | Slight cough, normal breath | Moderate cough, rapid breath, | Frequent cough, rapid panting | Chronic dry cough, weak to rapid breathing |
| **Nasal Discharge** | Normal, moist | Small amount of unilateral cloudy discharge | Bilateral, cloudy or excessive mucus discharge | Copious bilateral mucopurulent discharge | |
| |  |  |  |  | |

Picture source: https://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_health_scoring_chart.pdf

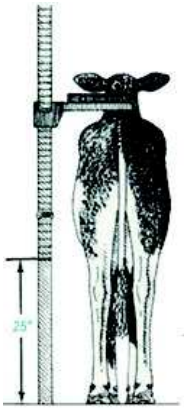
Fecal score: Pineda et al., JDS 2016; http://dx.doi.org/10.3168/jds.2016-11237

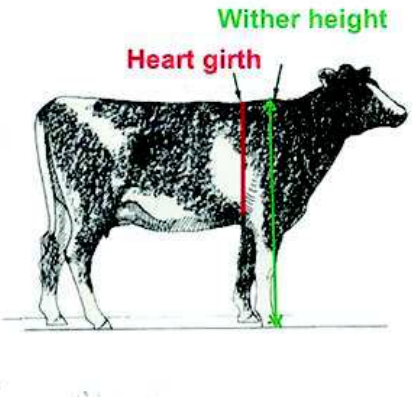
Respiratory score: http://extension.psu.edu/animals/dairy/nutrition/calves/calftrack/health-scoring-system-in-english

**Bodyframe measurements**

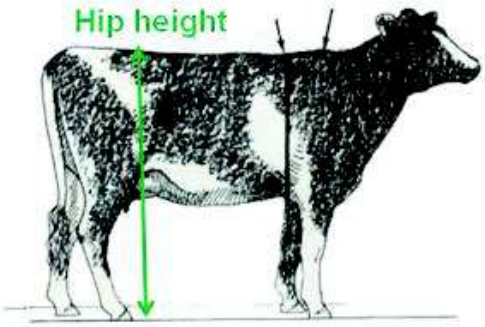
## Measure Body Frame of Dairy Calf

### 1. Wither Height and Heart Girth






### 2. Hip width and height




### 3. Body length (point of shoulder to the ischium)

## J1602: Weekly Body Frame Record

| ID | Trt | Arrival: Date | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Wither height | Body length | Heart girth | Hip height | Hip width | | | | | |
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**Milk replacer feeding table**

## Table: Volume of liquid MR for each calf per feeding

***UPDATED ON:*** ***EFFECTIVE UNITL:*** **Bottle / Bucket**

| Calf ID | BW | Vol. of MR, mL | | |
|---|---|---|---|---|
| | | CON | LOW | HIGH |
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- Day 1-3: MR volume per feeding, mL = (calf BW, kg × 0.01) ÷ 0.13
- Day 4-7: MR volume per feeding, mL = (calf BW, kg × 0.012) ÷ 0.13
- Wk 2-6: MR volume per feeding, mL = (calf BW, kg × 0.015) ÷ 0.13
- Wk 7: MR volume per feeding, mL = (calf BW, kg × 0.01) ÷ 0.13
- Wk 8: MR volume per feeding, mL = (calf BW, kg × 0.005) ÷ 0.13

**Milk replacer equations**

## Table: Group Milk Replacer Allowance at Each Feeding

***UPDATED ON:*** ***EFFECTIVE UNTIL:***

| Wk: Day: | CON | LOW | HIGH |
|---|---|---|---|
| Group BW, kg | | | |
| Group MR powder, g | | | |
| Group liquid MR volume, L | | | |
| # of 15-L bucket | | | |
| # of 10-L bucket | | | |
| # of 5-L bucket | | | |

**Equation for group MR powder at each feeding**

- **Day 1-3: MR powder, g = 0.5×(group BW,kg × 0.01)×1000 ×1.05**
- **Day 4-7: MR powder, g = 0.5×(group BW, kg × 0.012)×1000 ×1.05**
- **Wk 2-6: MR powder, g = 0.5×(group BW, kg × 0.015)×1000 ×1.05**
- **Wk 7: MR powder, g = 0.5×(group BW,kg × 0.01)×1000 ×1.05**
- **Wk 8: MR powder, g = 0.5×(group BW,kg × 0.005)×1000 ×1.05**

**Equation for group liquid MR volume**

- **Group liquid MR volume, L = Group MR powder, g ÷ 0.13**

**Work duties on each shift**

# SOP: Work Shift Duties

## Weekday AM shift: Start at 06:30 AM

- Intern 1: Perform health check and record score on datasheet and white board. Mark sign of sickness on the rear wall of hutches. Refill bedding materials
- Intern 2: Prepare, record and distribute milk, wash milk bottle/bucket with dish soap
- Intern 3: Measure/weigh and record refusal of water and starter grain; Wash water bucket; Prepare and distribute fresh water and starter grain.
- Intern 4: Help with feeding and everything else.

## Weekday PM shift: start at 04:30 PM

- Intern 1: Prepare, record and distribute milk, wash milk bottle/bucket
- Intern 2: Measure refusal water and wash water bucket; Prepare and distribute fresh water; Refill starter if the bucket is nearly empty and record on datasheet; Refill bedding materials if needed.
- Intern 3: help with feeding and other duties

## Weekend AM shift: start at 06:30 AM

- Intern 1: Perform health check and record score on datasheet and white board. Mark sign of sickness on the rear wall of hutches. Completely change bedding materials with help from other interns and spray 10% bleach on the old hutch location
- Intern 2: Prepare and distribute milk; Wash milk bottle/bucket with 10% bleach and rinse well
- Intern 3: Measure refusal water and starter grain; Wash water and starter grain bucket with 10% bleach and rinse well; Prepare and distribute fresh water and starter grain.
- Intern 4: Help with changing bedding material, feeding milk etc.

## Weekend PM shift: Start at 03:00 PM

- Intern 1,2,3, 4: Blood sampling collection and measure BW on Saturday; Measure body frame on Sunday
- Intern 1: Prepare, record and distribute milk, wash milk bottle/bucket
- Intern 2: Measure refusal water and wash water bucket; Prepare and distribute fresh water; Refill starter if the bucket is nearly empty and record on datasheet; Refill bedding materials if needed.
- Intern 3 and 4: help with feeding and everything else.

**Using the refractometer protocol**

# Using Refractometer Measure Serum Total Protein

## Calibration:

1. Wipe the window of refractometer with a tissue-paper.
2. Put 1 or 2 drop of distilled water on to the window of refractometer, and gently flip down the window cover. Make sure there is no air bubble enclosed and the whole window is covered by water.
3. Hold refractometer horizontally towards the light and look through eyepiece.
4. If the bright line of reading stay at "1.000" means no calibration is needed.
5. If the bright line of reading is NOT at "1.000", use the screw driver to adjust to "1.000" at the bottom of refractometer.
6. The optical component of refractometer change slightly with different temperature of the sample, so please use a distilled water with a room temperature (~21°C/70°F)

## Measure serum total protein:

1. Wipe the window of refractometer with a tissue-paper.
2. Put 1 or 2 drop of serum on to the window and gently flip down the cover. Make sure no air bubble enclosed and the whole window is covered by serum. (the blood was centrifuged at 20°C the same as room temperature)
3. Hold refractometer horizontally towards the light and look through eyepiece.
4. There should be clear and sharp separation between bright and dark area. Give the reading where the separation is based on the scale on the left side, which showed "serum protein" under the scale.
5. Write down the number in the calf datasheet.
6. Wipe off the serum on the window, and use 2 drops of distilled water to clean the window and wipe off.
7. Read the instructions in the box of refractometer, if there is any other problem observed.

**Mixing the milk replacer**

# SOP: Making Milk Replacer (MR) at Each Feeding

1. Always make milk replacer following the order of CON -> LOW -> HIGH group

2. Check the Table of "*Group Milk Replacer Allowance at Each Feeding*" to determine the total volume and number of 15-L, 10-L and/or 5-L unit you need to make for the group.

3. Check table below and weigh out the amount of MR powder for each 15-, 10- or 5-L liquid MR.

| **Final volume, L** | **15 L** | **10 L** | **5 L** |
|---|---|---|---|
| **MR powder, g** | **1950** | **1300** | **650** |

4. To make 15 L of MR, fill the 5-gallon bucket with half of the final volume of hot water (~140°F).

5. Dump powder into hot water and mixing for ~2 min.

**!!! 5.1. If this is for LOW or HIGH group, please dump one of corresponding tube of supplement into the bucket and mix for another min!!!**

6. Add cold water to the line of final volume (e.g. 15 L)

7. Cover the bucket and the rest volume needed for the group.

8. When all liquid MR is ready for the group, ship all 5-gallon buckets to hutches.

9. Check Table of "*Volume of liquid MR for each calf per feeding*"
Measured out the required volume for each calf using volumetric cylinder and distribute to each individual's water bucket.

10. Fill the bottle and feed calf.

## J1602 Starter Grain Intake & Refusal

**Week Starting Date:**

| ID | Trt | gram/day | AM Refusal & PM Refill (if empty), gram | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mon | | Tue | | Wed | | Thu | | Fri | | Sat | | Sun | |
| | | | AM | PM | AM | PM | AM | PM | AM | PM | AM | PM | AM | PM | AM | PM |
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# Appendix B

```
#Body weight
setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(lme4)
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
             startRow=1,
             cols=c(2:5))
#View(bw)

newBWW<-bw[ ! bw$Study.ID %in% c(6), ]
#View(newBWW)
newBW  <- na.omit(newBWW)
#View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)
#-------------------------SUBSETTING WEEK 0 DATA---------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
```

```
names(BWstart)[names(BWstart)=="BW"] <- "startbw"
#View(BWstart)

#-------------------------------WEEK 1-------------------------------
##########Subsetting data:
BW1 <- subset(newBW, WK == "1")
#View(BW1)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
anaBW1 <- merge(BW1, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaBW1)
nestID1<- anaBW1$TRT:anaBW1$Study.ID

model1 <- lme(BW~TRT+startbw+BW0,random = ~1|nestID1,method =
"ML",data=anaBW1,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID1, fixed = TRUE))
library(MASS)
a<-stepAIC(model1)
summary(a)
model1a<-lme(BW~startbw+BW0,random = ~1|nestID1,method =
"REML",data=anaBW1,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID1, fixed = TRUE))
summary(model1a)
anova(model1a)

#-------------------------------WEEK 2-------------------------------
##########Subsetting data:
BW2 <- subset(newBW, WK == "2")
#View(BW2)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
```

```
#View(BWstart1)
anaBW2 <- merge(BW2, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaBW2)
nestID2<- anaBW2$TRT:anaBW2$Study.ID

model2 <- lme(BW~TRT+startbw+BW0,random = ~1|nestID2,method =
"ML",data=anaBW2,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID2, fixed = TRUE))
library(MASS)
a<-stepAIC(model2)
summary(a)
model2a<-lme(BW~startbw+BW0,random = ~1|nestID2,method =
"REML",data=anaBW2,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID2, fixed = TRUE))
summary(model2a)
anova(model2a)
#------------------------------WEEK 3--------------------------------
##########Subsetting data:
BW3 <- subset(newBW, WK == "3")
#View(BW3)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
anaBW3 <- merge(BW3, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaBW3)
nestID3<- anaBW3$TRT:anaBW3$Study.ID

model3 <- lme(BW~TRT+startbw+BW0,random = ~1|nestID3,method =
"ML",data=anaBW3,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID3, fixed = TRUE))
library(MASS)
```

```
a<-stepAIC(model3)
summary(a)
model3a<-lme(BW~startbw,random = ~1|nestID3,method =
"REML",data=anaBW3,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID3, fixed = TRUE))
summary(model3a)
anova(model3a)
#---------------------------------WEEK 4-----------------------------
##########Subsetting data:
BW4 <- subset(newBW, WK == "4")
#View(BW4)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
anaBW4 <- merge(BW4, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaBW4)
nestID4<- anaBW4$TRT:anaBW4$Study.ID

model4 <- lme(BW~TRT+startbw+BW0,random = ~1|nestID4,method =
"ML",data=anaBW4,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID4, fixed = TRUE))
library(MASS)
a<-stepAIC(model4)
summary(a)
model4a<-lme(BW~TRT+BW0,random = ~1|nestID4,method =
"REML",data=anaBW4,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID4, fixed = TRUE))
summary(model4a)
anova(model4a)
########POSTHOC TUKEY TEST
library(multcomp)
A<-glht(model4a, linfct=mcp(TRT="Tukey"))
```

```
summary(A)
B<-cld(A,details=TRUE,letters=c("a,b,c"))
B
#------------------------------WEEK 5-------------------------------
##########Subsetting data:
BW5 <- subset(newBW, WK == "5")
#View(BW5)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
anaBW5 <- merge(BW5, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaBW5)
nestID5<- anaBW5$TRT:anaBW5$Study.ID

model5 <- lme(BW~TRT+startbw+BW0,random = ~1|nestID5,method =
"ML",data=anaBW5,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID5, fixed = TRUE))
library(MASS)
a<-stepAIC(model5)
summary(a)
model5a <-lme(BW~TRT+BW0,random = ~1|nestID5,method =
"REML",data=anaBW5,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID5, fixed = TRUE))
summary(model5a)
anova(model5a)
########POSTHOC TUKEY TEST
library(multcomp)
C<-glht(model5a, linfct=mcp(TRT="Tukey"))
summary(C)
D<-cld(C,details=TRUE,letters=c("a,b,c"))
D
#------------------------------WEEK 6-------------------------------
```

```
##########Subsetting data:
BW6 <- subset(newBW, WK == "6")
#View(BW6)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
anaBW6 <- merge(BW6, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaBW6)
nestID6<- anaBW6$TRT:anaBW6$Study.ID

model6 <- lme(BW~TRT+startbw+BW0,random = ~1|nestID6,method =
"ML",data=anaBW6,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID6, fixed = TRUE))
library(MASS)
a<-stepAIC(model6)
summary(a)
model6a <- lme(BW~TRT+startbw,random = ~1|nestID6,method =
"REML",data=anaBW6,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID6, fixed = TRUE))
summary(model6a)
anova(model6a)
########POSTHOC TUKEY TEST
library(multcomp)
E<-glht(model6a, linfct=mcp(TRT="Tukey"))
summary(E)
G<-cld(E,details=TRUE,letters=c("a,b,c"))
G
#------------------------------WEEK 7--------------------------------
##########Subsetting data:
BW7 <- subset(newBW, WK == "7")
#View(BW7)
drops <- c("WK")
```

```
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
anaBW7 <- merge(BW7, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaBW7)
nestID7<- anaBW7$TRT:anaBW7$Study.ID

model7 <- lme(BW~TRT+startbw+BW0,random = ~1|nestID7,method =
"ML",data=anaBW7,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID7, fixed = TRUE))
library(MASS)
a<-stepAIC(model7)
summary(a)
model7a <- lme(BW~startbw,random = ~1|nestID7,method =
"REML",data=anaBW7,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID7, fixed = TRUE))
summary(model7a)
anova(model7a)
#--------------------------------WEEK 8-----------------------------
##########Subsetting data:
BW8 <- subset(newBW, WK == "8")
#View(BW8)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
anaBW8 <- merge(BW8, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaBW8)
nestID8<- anaBW8$TRT:anaBW8$Study.ID

model8 <- lme(BW~TRT+startbw+BW0,random = ~1|nestID8,method =
"ML",data=anaBW8,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID8, fixed = TRUE))
```

```
library(MASS)
a<-stepAIC(model8)
summary(a)
model8a <- lme(BW~startbw,random = ~1|nestID8,method =
"REML",data=anaBW8,na.action=na.omit, corr = corCAR1(form = ~ WK |
nestID8, fixed = TRUE))
summary(model8a)
anova(model8a)

aggregate(newBW$BW, by=list(newBW$TRT, newBW$WK), FUN=mean,na.rm=TRUE)
aggregate(newBW$BW, by=list(newBW$TRT, newBW$WK), FUN=sd,na.rm=TRUE)
```

```
#Body length

setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(lme4)
library(multcomp)

#BODYMEASUREMENTS
bodymeasurements=read.xlsx(xlsxFile="production_data_april.xlsx",
                           sheet=7,
                           startRow=1,
                           cols=c(2:9))
#View(bodymeasurements)

nnewBM<-bodymeasurements[ ! bodymeasurements$Study.ID %in% c(6), ]
#View(nnewBM)


newBM <- nnewBM[ ! nnewBM$Wk %in% c(1), ]
#View(newBM)
is.data.frame(newBM)
is.numeric(newBM$`body.length.cm`)
newBM$Study.ID <- factor(newBM$Study.ID)
is.factor(newBM$Study.ID)
newBM$TRT<- factor(newBM$TRT)
is.factor(newBM$TRT)
newBM$Wk<- factor(newBM$Wk)
is.factor(newBM$Wk)
is.numeric(newBM$Wk)
levels(newBM$Wk)
#_____________________________________LOADING BW_____________________________
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
```

```
              sheet=2,
              startRow=1,
              cols=c(2:5))
#View(bw)

newBWW<-bw[ ! bw$Study.ID %in% c(6), ]
#View(newBWW)
newBW  <- na.omit(newBWW)
#View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)
#--------------------------SUBSETTING WEEK 0 DATA--------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
levels(BWstart$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
#View(BWstart)

BMstart <- subset(newBM, Wk == "0")
#View(BMstart)

analysisBM <- merge(BMstart, BWstart, by.x=c("Study.ID","TRT","Wk"),
by.y=c("Study.ID","TRT","WK"),all=TRUE,all.x=TRUE)
```

```
#View(analysisBM)
analysisBM
analysisBM$BW0<-factor(analysisBM$BW0)
is.factor(analysisBM$BWO)
write.table(analysisBM, "analysis_BM.txt", sep="\t", quote=F)
analysisBM$ BW0 <- as.numeric(analysisBM$ BW0)
str(analysisBM)
head(analysisBM)
summary(analysisBM)
#_____________________________CORRELATION_____________________________
cor.test(x=analysisBM$body.length.cm,y=analysisBM$BW,method =
"pearson")

#------------------------------WEEK 0-------------------------------
##########Subsetting data:
BM0raw <- subset(newBM, Wk == "0")
View(BM0raw)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
anaBM0 <- merge(BM0raw, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM0)
nestID0<- anaBM0$TRT:anaBM0$Study.ID

model0 <- lme(body.length.cm~TRT+BW+BW0,random = ~1|nestID0,method =
"ML",data=anaBM0,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID0, fixed = TRUE))
library(MASS)
a<-stepAIC(model0)
summary(a)
model0a <- lme(body.length.cm~BW,random = ~1|nestID0,method =
"REML",data=anaBM0,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID0, fixed = TRUE))
```

```
summary(model0a)
anova(model0a)
#------------------------------WEEK 4--------------------------------
##########Subsetting data:
BM4 <- subset(newBM, Wk == "4")
View(BM4)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
View(BWstart1)
anaBM4 <- merge(BM4, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM4)
nestID4<- anaBM4$TRT:anaBM4$Study.ID

model4 <- lme(body.length.cm~TRT+BW+BW0,random = ~1|nestID4,method =
"ML",data=anaBM4,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID4, fixed = TRUE))
library(MASS)
a<-stepAIC(model4)
summary(a)
model4a <-lme(body.length.cm~TRT+BW,random = ~1|nestID4,method =
"REML",data=anaBM4,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID4, fixed = TRUE))
summary(model4a)
anova(model4a)
########POSTHOC TUKEY TEST
A1<-glht(model4a, linfct=mcp(TRT="Tukey"))
summary(A1)
B1<-cld(A1,details=TRUE,letters=c("a,b,c"))
B1
#-----------------------------WEEK 6---------------------------------
##########Subsetting data:
BM6 <- subset(newBM, Wk == "6")
```

```
View(BM6)
drops <- c("WK")
anaBM6 <- merge(BM6, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM6)
nestID6<- anaBM6$TRT:anaBM6$Study.ID

model6 <- lme(body.length.cm~TRT+BW+BW0,random = ~1|nestID6,method =
"ML",data=anaBM6,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID6, fixed = TRUE))
library(MASS)
a<-stepAIC(model6)
summary(a)
model6a <- lme(body.length.cm~BW,random = ~1|nestID6,method =
"REML",data=anaBM6,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID6, fixed = TRUE))
summary(model6a)
anova(model6a)
#-----------------------------WEEK 8---------------------------------
##########Subsetting data:
BM8 <- subset(newBM, Wk == "8")
View(BM8)
drops <- c("WK")
anaBM8 <- merge(BM8, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM8)
nestID8<- anaBM8$TRT:anaBM8$Study.ID

model8 <- lme(body.length.cm~TRT+BW+BW0,random = ~1|nestID8,method =
"ML",data=anaBM8,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID8, fixed = TRUE))
library(MASS)
a<-stepAIC(model8)
```

```
summary(a)
model8a <- lme(body.length.cm~BW,random = ~1|nestID8,method =
"REML",data=anaBM8,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID8, fixed = TRUE))
summary(model8a)
anova(model8a)

aggregate(newBM$body.length.cm, by=list(newBM$TRT, newBM$Wk),
FUN=mean, na.rm=TRUE)
aggregate(newBM$body.length.cm, by=list(newBM$TRT, newBM$Wk), FUN=sd,
na.rm=TRUE)
```

```
#Hip width

setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(multcomp)

#BODYMEASUREMENTS
bodymeasurements=read.xlsx(xlsxFile="production_data_april.xlsx",
                           sheet=7,
                           startRow=1,
                           cols=c(2:9))
#View(bodymeasurements)

nnewBM<-bodymeasurements[ ! bodymeasurements$Study.ID %in% c(6), ]
#View(nnewBM)


newBM <- nnewBM[ ! nnewBM$Wk %in% c(1), ]
#View(newBM)
is.data.frame(newBM)
is.numeric(newBM$`hip.width.cm`)
newBM$Study.ID <- factor(newBM$Study.ID)
is.factor(newBM$Study.ID)
newBM$TRT<- factor(newBM$TRT)
is.factor(newBM$TRT)
newBM$Wk<- factor(newBM$Wk)
is.factor(newBM$Wk)
is.numeric(newBM$Wk)
levels(newBM$Wk)
#_____________________________LOADING BW_____________________________
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
```

```
             startRow=1,
             cols=c(2:5))
#View(bw)

newBWW<-bw[ ! bw$Study.ID %in% c(6), ]
#View(newBWW)
newBW  <- na.omit(newBWW)
#View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)
#-------------------------SUBSETTING WEEK 0 DATA---------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
levels(BWstart$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
#View(BWstart)

BMstart <- subset(newBM, Wk == "0")
#View(BMstart)

analysisBM <- merge(BMstart, BWstart, by.x=c("Study.ID","TRT","Wk"),
by.y=c("Study.ID","TRT","WK"),all=TRUE,all.x=TRUE)
#View(analysisBM)
```

```
analysisBM
analysisBM$BW0<-factor(analysisBM$BW0)
is.factor(analysisBM$BWO)
write.table(analysisBM, "analysis_BM.txt", sep="\t", quote=F)
analysisBM$ BW0 <- as.numeric(analysisBM$ BW0)
str(analysisBM)
head(analysisBM)
summary(analysisBM)
#_______________________________CORRELATION_______________________________
cor.test(x=analysisBM$hip.width.cm,y=analysisBM$BW,method = "pearson")

#-----------------------------WEEK 0---------------------------------
##########Subsetting data:
BM0raw <- subset(newBM, Wk == "0")
View(BM0raw)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
anaBM0 <- merge(BM0raw, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM0)
nestID0<- anaBM0$TRT:anaBM0$Study.ID

model0 <- lme(hip.width.cm~TRT+BW+BW0,random = ~1|nestID0,method =
"ML",data=anaBM0,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID0, fixed = TRUE))
library(MASS)
a<-stepAIC(model0)
summary(a)
model0a <- lme(hip.width.cm~TRT+BW,random = ~1|nestID0,method =
"REML",data=anaBM0,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID0, fixed = TRUE))
summary(model0a)
anova(model0a)
```

```
#------------------------------WEEK 4-------------------------------
##########Subsetting data:
BM4raw <- subset(newBM, Wk == "4")
View(BM4raw)
BW4raw <- subset(newBW, WK == "4")
levels(BW4raw$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
View(BW4raw)
drop <- c("WK")
BW4 <- BW4raw[ , !(names(BW4raw) %in% drop)]
BM4 <- merge(BM4raw, BW4, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
names(BM4)[names(BM4) == 'BW'] <- 'BW4'
View(BM4)

drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
View(BWstart1)
anaBM4 <- merge(BM4, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM4)
nestID4<- anaBM4$TRT:anaBM4$Study.ID

model4 <- lme(hip.width.cm~TRT+BW+BW0+BW4,random = ~1|nestID4,method =
"ML",data=anaBM4,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID4, fixed = TRUE))
library(MASS)
a<-stepAIC(model4)
summary(a)
model4a <- lme(hip.width.cm~BW0,random = ~1|nestID4,method =
"REML",data=anaBM4,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID4, fixed = TRUE))
summary(model4a)
anova(model4a)
```

```
#---------------------------------WEEK 6-----------------------------
##########Subsetting data:
BM6raw <- subset(newBM, Wk == "6")
View(BM6)
BW6raw <- subset(newBW, WK == "6")
levels(BW6raw$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
View(BW6raw)
drop <- c("WK")
BW6 <- BW6raw[ , !(names(BW6raw) %in% drop)]
BM6 <- merge(BM6raw, BW6, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
names(BM6)[names(BM6) == 'BW'] <- 'BW6'
View(BM6)

anaBM6 <- merge(BM6, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM6)
nestID6<- anaBM6$TRT:anaBM6$Study.ID

model6 <- lme(hip.width.cm~TRT+BW+BW0+BW6,random = ~1|nestID6,method =
"ML",data=anaBM6,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID6, fixed = TRUE))
library(MASS)
a<-stepAIC(model6)
summary(a)
model6a <- lme(hip.width.cm~TRT+BW0,random = ~1|nestID6,method =
"REML",data=anaBM6,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID6, fixed = TRUE))
summary(model6a)
anova(model6a)
########POSTHOC TUKEY TEST
A<-glht(model6a, linfct=mcp(TRT="Tukey"))
```

```
summary(A)
B<-cld(A,details=TRUE,letters=c("a,b,c"))
B
#---------------------------------WEEK 8-----------------------------
##########Subsetting data:
BM8raw <- subset(newBM, Wk == "8")
View(BM8)
BW8raw <- subset(newBW, WK == "8")
levels(BW8raw$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
View(BW8raw)
drop <- c("WK")
BW8 <- BW8raw[ , !(names(BW8raw) %in% drop)]
BM8 <- merge(BM8raw, BW8, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
names(BM8)[names(BM8) == 'BW'] <- 'BW8'
View(BM8)

anaBM8 <- merge(BM8, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM8)
nestID8<- anaBM8$TRT:anaBM8$Study.ID

model8 <- lme(hip.width.cm~TRT+BW+BW0+BW8,random = ~1|nestID8,method =
"ML",data=anaBM8,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID8, fixed = TRUE))
library(MASS)
a<-stepAIC(model8)
summary(a)
model8a <- lme(hip.width.cm~TRT+BW+BW0,random = ~1|nestID8,method =
"REML",data=anaBM8,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID8, fixed = TRUE))
summary(model8a)
anova(model8a)
```

```
########POSTHOC TUKEY TEST
C<-glht(model8a, linfct=mcp(TRT="Tukey"))
summary(C)
D<-cld(C,details=TRUE,letters=c("a,b,c"))
D


aggregate(newBM$hip.width.cm, by=list(newBM$TRT,newBM$Wk),
FUN=mean,na.rm=TRUE)
aggregate(newBM$hip.width.cm, by=list(newBM$TRT,newBM$Wk),
FUN=sd,na.rm=TRUE)
```

```
#Heart girth

setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(lme4)
library(multcomp)

#BODYMEASUREMENTS
bodymeasurements=read.xlsx(xlsxFile="production_data_april.xlsx",
                           sheet=7,
                           startRow=1,
                           cols=c(2:9))
#View(bodymeasurements)

nnewBM<-bodymeasurements[ ! bodymeasurements$Study.ID %in% c(6), ]
#View(nnewBM)


newBM <- nnewBM[ ! nnewBM$Wk %in% c(1), ]
#View(newBM)
is.data.frame(newBM)
is.numeric(newBM$`heart.girth.cm`)
newBM$Study.ID <- factor(newBM$Study.ID)
is.factor(newBM$Study.ID)
newBM$TRT<- factor(newBM$TRT)
is.factor(newBM$TRT)
newBM$Wk<- factor(newBM$Wk)
is.factor(newBM$Wk)
is.numeric(newBM$Wk)
levels(newBM$Wk)
#_____________________________________LOADING BW_____________________________
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
```

```
              sheet=2,
              startRow=1,
              cols=c(2:5))
#View(bw)

newBWW<-bw[ ! bw$Study.ID %in% c(6), ]
#View(newBWW)
newBW  <- na.omit(newBWW)
#View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)
#-----------------------SUBSETTING WEEK 0 DATA----------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
levels(BWstart$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
#View(BWstart)

BMstart <- subset(newBM, Wk == "0")
#View(BMstart)

analysisBM <- merge(BMstart, BWstart, by.x=c("Study.ID","TRT","Wk"),
by.y=c("Study.ID","TRT","WK"),all=TRUE,all.x=TRUE)
```

```
#View(analysisBM)
analysisBM
analysisBM$BW0<-factor(analysisBM$BW0)
is.factor(analysisBM$BWO)
write.table(analysisBM, "analysis_BM.txt", sep="\t", quote=F)
analysisBM$ BW0 <- as.numeric(analysisBM$ BW0)
str(analysisBM)
head(analysisBM)
summary(analysisBM)
#_______________________________CORRELATION_____________________________
cor.test(x=analysisBM$heart.girth.cm,y=analysisBM$BW,method =
"pearson")

#---------------------------------WEEK 0-----------------------------
##########Subsetting data:
BM0raw <- subset(newBM, Wk == "0")
View(BM0raw)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
anaBM0 <- merge(BM0raw, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM0)
nestID0<- anaBM0$TRT:anaBM0$Study.ID

model0 <- lme(heart.girth.cm~TRT+BW+BW0,random = ~1|nestID0,method =
"ML",data=anaBM0,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID0, fixed = TRUE))
library(MASS)
a<-stepAIC(model0)
summary(a)
model0a <- lme(heart.girth.cm~BW,random = ~1|nestID0,method =
"REML",data=anaBM0,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID0, fixed = TRUE))
```

```
summary(model0a)
anova(model0a)
#-------------------------------WEEK 4-------------------------------
##########Subsetting data:
BM4 <- subset(newBM, Wk == "4")
View(BM4)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
View(BWstart1)
anaBM4 <- merge(BM4, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM4)
nestID4<- anaBM4$TRT:anaBM4$Study.ID

model4 <- lme(heart.girth.cm~TRT+BW+BW0,random = ~1|nestID4,method =
"ML",data=anaBM4,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID4, fixed = TRUE))
library(MASS)
a<-stepAIC(model4)
summary(a)
model4a <- lme(heart.girth.cm~BW0,random = ~1|nestID4,method =
"REML",data=anaBM4,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID4, fixed = TRUE))
summary(model4a)
anova(model4a)

#-------------------------------WEEK 6-------------------------------
##########Subsetting data:
BM6 <- subset(newBM, Wk == "6")
View(BM6)
drops <- c("WK")
anaBM6 <- merge(BM6, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
```

```
View(anaBM6)
nestID6<- anaBM6$TRT:anaBM6$Study.ID

model6 <- lme(heart.girth.cm~TRT+BW+BW0,random = ~1|nestID6,method =
"ML",data=anaBM6,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID6, fixed = TRUE))
library(MASS)
a<-stepAIC(model6)
summary(a)
model6a <- lme(heart.girth.cm~TRT+BW0,random = ~1|nestID6,method =
"REML",data=anaBM6,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID6, fixed = TRUE))
summary(model6a)
anova(model6a)
########POSTHOC TUKEY TEST
A<-glht(model6a, linfct=mcp(TRT="Tukey"))
summary(A)
B<-cld(A,details=TRUE,letters=c("a,b,c"))
B
#------------------------------WEEK 8--------------------------------
##########Subsetting data:
BM8 <- subset(newBM, Wk == "8")
View(BM8)
drops <- c("WK")
anaBM8 <- merge(BM8, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM8)
nestID8<- anaBM8$TRT:anaBM8$Study.ID

model8 <- lme(heart.girth.cm~TRT+BW+BW0,random = ~1|nestID8,method =
"ML",data=anaBM8,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID8, fixed = TRUE))
library(MASS)
```

```
a<-stepAIC(model8)
summary(a)
model8a <- lme(heart.girth.cm~BW+BW0,random = ~1|nestID8,method =
"REML",data=anaBM8,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID8, fixed = TRUE))
summary(model8a)
anova(model8a)

str(anaBM8)
aggregate(newBM$heart.girth.cm, by=list(newBM$TRT, newBM$Wk),
FUN=mean,na.rm=TRUE)
aggregate(newBM$heart.girth.cm, by=list(newBM$TRT, newBM$Wk),
FUN=sd,na.rm=TRUE)
```

```
#Hip heigth

setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(lme4)
library(multcomp)

#BODYMEASUREMENTS
bodymeasurements=read.xlsx(xlsxFile="production_data_april.xlsx",
                           sheet=7,
                           startRow=1,
                           cols=c(2:9))
#View(bodymeasurements)

nnewBM<-bodymeasurements[ ! bodymeasurements$Study.ID %in% c(6), ]
#View(nnewBM)


newBM <- nnewBM[ ! nnewBM$Wk %in% c(1), ]
#View(newBM)
is.data.frame(newBM)
is.numeric(newBM$`hip.height.cm`)
newBM$Study.ID <- factor(newBM$Study.ID)
is.factor(newBM$Study.ID)
newBM$TRT<- factor(newBM$TRT)
is.factor(newBM$TRT)
newBM$Wk<- factor(newBM$Wk)
is.factor(newBM$Wk)
is.numeric(newBM$Wk)
levels(newBM$Wk)
#_____________________________________LOADING BW_____________________________
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
```

```
              sheet=2,
              startRow=1,
              cols=c(2:5))
#View(bw)

newBWW<-bw[ ! bw$Study.ID %in% c(6), ]
#View(newBWW)
newBW  <- na.omit(newBWW)
#View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)
#-----------------------SUBSETTING WEEK 0 DATA----------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
levels(BWstart$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
#View(BWstart)

BMstart <- subset(newBM, Wk == "0")
#View(BMstart)

analysisBM <- merge(BMstart, BWstart, by.x=c("Study.ID","TRT","Wk"),
by.y=c("Study.ID","TRT","WK"),all=TRUE,all.x=TRUE)
```

```
#View(analysisBM)
analysisBM
analysisBM$BW0<-factor(analysisBM$BW0)
is.factor(analysisBM$BWO)
write.table(analysisBM, "analysis_BM.txt", sep="\t", quote=F)
analysisBM$ BW0 <- as.numeric(analysisBM$ BW0)
str(analysisBM)
head(analysisBM)
summary(analysisBM)
#_____________________________CORRELATION_____________________________
cor.test(x=analysisBM$hip.height.cm,y=analysisBM$BW,method =
"pearson")

#----------------------------WEEK 0---------------------------------
##########Subsetting data:
BM0raw <- subset(newBM, Wk == "0")
View(BM0raw)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
anaBM0 <- merge(BM0raw, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM0)
nestID0<- anaBM0$TRT:anaBM0$Study.ID

model0 <- lme(hip.height.cm~TRT+BW+BW0,random = ~1|nestID0,method =
"ML",data=anaBM0,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID0, fixed = TRUE))
library(MASS)
a<-stepAIC(model0)
summary(a)
model0a <- lme(hip.height.cm~BW+BW0,random = ~1|nestID0,method =
"REML",data=anaBM0,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID0, fixed = TRUE))
```

```
summary(model0a)
anova(model0a)
#-------------------------------WEEK 4-------------------------------
##########Subsetting data:
BM4raw <- subset(newBM, Wk == "4")
View(BM4raw)
BW4raw <- subset(newBW, WK == "4")
levels(BW4raw$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
View(BW4raw)
drop <- c("WK")
BW4 <- BW4raw[ , !(names(BW4raw) %in% drop)]
BM4 <- merge(BM4raw, BW4, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
names(BM4)[names(BM4) == 'BW'] <- 'BW4'
View(BM4)

drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
View(BWstart1)
anaBM4 <- merge(BM4, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM4)
nestID4<- anaBM4$TRT:anaBM4$Study.ID

model4 <- lme(hip.height.cm~TRT+BW+BW0+BW4,random = ~1|nestID4,method
= "ML",data=anaBM4,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID4, fixed = TRUE))
library(MASS)
a<-stepAIC(model4)
summary(a)
model4a <- lme(hip.height.cm~TRT+BW4,random = ~1|nestID4,method =
"REML",data=anaBM4,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID4, fixed = TRUE))
```

```
summary(model4a)
anova(model4a)
########POSTHOC TUKEY TEST
library(multcomp)
A1<-glht(model4a, linfct=mcp(TRT="Tukey"))
summary(A1)
B1<-cld(A1,details=TRUE,letters=c("a,b,c"))
B1
#--------------------------WEEK 6-----------------------------------
##########Subsetting data:
BM6raw <- subset(newBM, Wk == "6")
View(BM6)
BW6raw <- subset(newBW, WK == "6")
levels(BW6raw$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
View(BW6raw)
drop <- c("WK")
BW6 <- BW6raw[ , !(names(BW6raw) %in% drop)]
BM6 <- merge(BM6raw, BW6, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
names(BM6)[names(BM6) == 'BW'] <- 'BW6'
View(BM6)

anaBM6 <- merge(BM6, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM6)
nestID6<- anaBM6$TRT:anaBM6$Study.ID

model6 <- lme(hip.height.cm~TRT+BW+BW0+BW6,random = ~1|nestID6,method
= "ML",data=anaBM6,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID6, fixed = TRUE))
library(MASS)
a<-stepAIC(model6)
summary(a)
```

```
model6a <- lme(hip.height.cm~TRT+BW6,random = ~1|nestID6,method =
"REML",data=anaBM6,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID6, fixed = TRUE))
summary(model6a)
anova(model6a)
########POSTHOC TUKEY TEST
A<-glht(model6a, linfct=mcp(TRT="Tukey"))
summary(A)
B<-cld(A,details=TRUE,letters=c("a,b,c"))
B
#--------------------------------WEEK 8-------------------------------
##########Subsetting data:
BM8raw <- subset(newBM, Wk == "8")
View(BM8)
BW8raw <- subset(newBW, WK == "8")
levels(BW8raw$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
View(BW8raw)
drop <- c("WK")
BW8 <- BW8raw[ , !(names(BW8raw) %in% drop)]
BM8 <- merge(BM8raw, BW8, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
names(BM8)[names(BM8) == 'BW'] <- 'BW8'
View(BM8)

anaBM8 <- merge(BM8, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM8)
nestID8<- anaBM8$TRT:anaBM8$Study.ID

model8 <- lme(hip.height.cm~TRT+BW+BW0+BW8,random = ~1|nestID8,method
= "ML",data=anaBM8,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID8, fixed = TRUE))
library(MASS)
```

```
a<-stepAIC(model8)
summary(a)
model8a <- lme(hip.height.cm~TRT+BW+BW8,random = ~1|nestID8,method =
"REML",data=anaBM8,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID8, fixed = TRUE))
summary(model8a)
anova(model8a)
########POSTHOC TUKEY TEST
C<-glht(model8a, linfct=mcp(TRT="Tukey"))
summary(C)
D<-cld(C,details=TRUE,letters=c("a,b,c"))
D

aggregate(newBM$hip.height.cm, by=list(newBM$TRT,newBM$Wk),
FUN=mean,na.rm=TRUE)
aggregate(newBM$hip.height.cm, by=list(newBM$TRT,newBM$Wk),
FUN=sd,na.rm=TRUE)
```

```
#Wither height

setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(multcomp)

#BODYMEASUREMENTS
bodymeasurements=read.xlsx(xlsxFile="production_data_april.xlsx",
                           sheet=7,
                           startRow=1,
                           cols=c(2:9))
#View(bodymeasurements)

nnewBM<-bodymeasurements[ ! bodymeasurements$Study.ID %in% c(6), ]
#View(nnewBM)


newBM <- nnewBM[ ! nnewBM$Wk %in% c(1), ]
#View(newBM)
is.data.frame(newBM)
is.numeric(newBM$`wither.height.cm`)
newBM$Study.ID <- factor(newBM$Study.ID)
is.factor(newBM$Study.ID)
newBM$TRT<- factor(newBM$TRT)
is.factor(newBM$TRT)
newBM$Wk<- factor(newBM$Wk)
is.factor(newBM$Wk)
is.numeric(newBM$Wk)
levels(newBM$Wk)
#_____________________________LOADING BW_____________________________
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
```

```
             startRow=1,
             cols=c(2:5))
#View(bw)

newBWW<-bw[ ! bw$Study.ID %in% c(6), ]
#View(newBWW)
newBW  <- na.omit(newBWW)
#View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)
#-----------------------SUBSETTING WEEK 0 DATA----------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
levels(BWstart$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
#View(BWstart)

BMstart <- subset(newBM, Wk == "0")
#View(BMstart)

analysisBM <- merge(BMstart, BWstart, by.x=c("Study.ID","TRT","Wk"),
by.y=c("Study.ID","TRT","WK"),all=TRUE,all.x=TRUE)
#View(analysisBM)
```

```
analysisBM
analysisBM$BW0<-factor(analysisBM$BW0)
is.factor(analysisBM$BWO)
write.table(analysisBM, "analysis_BM.txt", sep="\t", quote=F)
analysisBM$ BW0 <- as.numeric(analysisBM$ BW0)
str(analysisBM)
head(analysisBM)
summary(analysisBM)
#_____________________________CORRELATION_____________________________
cor.test(x=analysisBM$wither.height.cm,y=analysisBM$BW,method =
"pearson")

#-------------------------------WEEK 0-------------------------------
##########Subsetting data:
BM0raw <- subset(newBM, Wk == "0")
View(BM0raw)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
anaBM0 <- merge(BM0raw, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM0)
nestID0<- anaBM0$TRT:anaBM0$Study.ID

model0 <- lme(wither.height.cm~TRT+BW+BW0,random = ~1|nestID0,method =
"ML",data=anaBM0,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID0, fixed = TRUE))
library(MASS)
a<-stepAIC(model0)
summary(a)
model0a <- lme(wither.height.cm~BW+BW0,random = ~1|nestID0,method =
"REML",data=anaBM0,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID0, fixed = TRUE))
summary(model0a)
```

```
anova(model0a)
#------------------------------WEEK 4---------------------------------
##########Subsetting data:
BM4raw <- subset(newBM, Wk == "4")
View(BM4raw)
BW4raw <- subset(newBW, WK == "4")
levels(BW4raw$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
View(BW4raw)
drop <- c("WK")
BW4 <- BW4raw[ , !(names(BW4raw) %in% drop)]
BM4 <- merge(BM4raw, BW4, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
names(BM4)[names(BM4) == 'BW'] <- 'BW4'
View(BM4)

drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
View(BWstart1)
anaBM4 <- merge(BM4, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM4)
nestID4<- anaBM4$TRT:anaBM4$Study.ID

model4 <- lme(wither.height.cm~TRT+BW+BW0+BW4,random =
~1|nestID4,method = "ML",data=anaBM4,na.action=na.omit, corr =
corCAR1(form = ~ Wk | nestID4, fixed = TRUE))
library(MASS)
a<-stepAIC(model4)
summary(a)
model4a <- lme(wither.height.cm~BW4,random = ~1|nestID4,method =
"REML",data=anaBM4,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID4, fixed = TRUE))
summary(model4a)
```

```
anova(model4a)
#------------------------------WEEK 6--------------------------------
##########Subsetting data:
BM6raw <- subset(newBM, Wk == "6")
View(BM6)
BW6raw <- subset(newBW, WK == "6")
levels(BW6raw$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
View(BW6raw)
drop <- c("WK")
BW6 <- BW6raw[ , !(names(BW6raw) %in% drop)]
BM6 <- merge(BM6raw, BW6, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
names(BM6)[names(BM6) == 'BW'] <- 'BW6'
View(BM6)

anaBM6 <- merge(BM6, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM6)
nestID6<- anaBM6$TRT:anaBM6$Study.ID

model6 <- lme(wither.height.cm~TRT+BW+BW0+BW6,random =
~1|nestID6,method = "ML",data=anaBM6,na.action=na.omit, corr =
corCAR1(form = ~ Wk | nestID6, fixed = TRUE))
library(MASS)
a<-stepAIC(model6)
summary(a)
model6a <- lme(wither.height.cm~BW6,random = ~1|nestID6,method =
"REML",data=anaBM6,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID6, fixed = TRUE))
summary(model6a)
anova(model6a)

#------------------------------WEEK 8--------------------------------
```

```
##########Subsetting data:
BM8raw <- subset(newBM, Wk == "8")
View(BM8)
BW8raw <- subset(newBW, WK == "8")
levels(BW8raw$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
View(BW8raw)
drop <- c("WK")
BW8 <- BW8raw[ , !(names(BW8raw) %in% drop)]
BM8 <- merge(BM8raw, BW8, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
names(BM8)[names(BM8) == 'BW'] <- 'BW8'
View(BM8)

anaBM8 <- merge(BM8, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaBM8)
nestID8<- anaBM8$TRT:anaBM8$Study.ID

model8 <- lme(wither.height.cm~TRT+BW+BW0+BW8,random =
~1|nestID8,method = "ML",data=anaBM8,na.action=na.omit, corr =
corCAR1(form = ~ Wk | nestID8, fixed = TRUE))
library(MASS)
a<-stepAIC(model8)
summary(a)
model8a <- lme(wither.height.cm~TRT+BW+BW8,random = ~1|nestID8,method
= "REML",data=anaBM8,na.action=na.omit, corr = corCAR1(form = ~ Wk |
nestID8, fixed = TRUE))
summary(model8a)
anova(model8a)
########POSTHOC TUKEY TEST
C<-glht(model8a, linfct=mcp(TRT="Tukey"))
summary(C)
D<-cld(C,details=TRUE,letters=c("a,b,c"))
```

D

```
aggregate(newBM$wither.height.cm, by=list(newBM$TRT, newBM$Wk),
FUN=mean,na.rm=TRUE)
aggregate(newBM$wither.height.cm, by=list(newBM$TRT, newBM$Wk),
FUN=sd,na.rm=TRUE)
```

```
# STARTER GRAIN
setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(lme4)

SG=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=6,
             startRow=1,
             cols=c(2:6))
#View(SG)

#whitout outliers:
SGO=read.xlsx(xlsxFile="production_data_april.xlsx",
              sheet=6,
              startRow=1,
              cols=c(11:15))
#View(SGO)

newSGO  <- na.omit(SGO)
#View(newSGO)
is.numeric(newSGO$"Grain.intake,.g")
newSGO$Day<- factor(newSGO$Day)
is.factor(newSGO$Day)
newSGO$wk<- factor(newSGO$wk)
is.factor(newSGO$wk)
newSGO$Study.ID <- factor(newSGO$Study.ID)
is.factor(newSGO$Study.ID)
newSGO$TRT<- factor(newSGO$TRT)
is.factor(newSGO$TRT)
levels(newSGO$wk)
levels(newSGO$TRT)
levels(newSGO$Study.ID)
```

```
nestID<- newSGO$TRT:newSGO$Study.ID
levels(nestID)
##-----------------------------BW-data-------------------------------
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
               sheet=2,
               startRow=1,
               cols=c(2:5))
#View(bw)

newBW  <- na.omit(bw)
#View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)
#-----------------------SUBSETTING WEEK 0 DATA----------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
names(BWstart)[names(BWstart)=="BW"] <- "startbw"
#View(BWstart)

#---------------------------WEEK 1-----------------------------------
##########Subsetting data:
```

```
SGO1 <- subset(newSGO, wk == "1")
#View(SGO1)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaSGO1 <- merge(SGO1, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaSGO1)
nestID1<- anaSGO1$TRT:anaSGO1$Study.ID

model1 <- lme(Grain.intake.g~TRT+startbw+BW0,random =
~1|nestID1,method = "ML",data=anaSGO1,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID1, fixed = TRUE))
library(MASS)
a<-stepAIC(model1)
summary(a)
model1a <- lme(Grain.intake.g~startbw,random = ~1|nestID1,method =
"REML",data=anaSGO1,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID1, fixed = TRUE))
summary(model1a)
anova(model1a)
#------------------------------WEEK 2--------------------------------
##########Subsetting data:
SGO2 <- subset(newSGO, wk == "2")
#View(SGO2)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaSGO2 <- merge(SGO2, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaSGO2)
```

```
nestID2<- anaSGO2$TRT:anaSGO2$Study.ID

model2 <- lme(Grain.intake.g~TRT+startbw+BW0,random =
~1|nestID2,method = "ML",data=anaSGO2,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID2, fixed = TRUE))
library(MASS)
a<-stepAIC(model2)
summary(a)
model2a <- lme(Grain.intake.g~BW0,random = ~1|nestID2,method =
"REML",data=anaSGO2,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID2, fixed = TRUE))
summary(model2a)
anova(model2a)
#-------------------------------WEEK 3-------------------------------
##########Subsetting data:
SGO3 <- subset(newSGO, wk == "3")
#View(SGO3)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaSGO3 <- merge(SGO3, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaSGO3)
nestID3<- anaSGO3$TRT:anaSGO3$Study.ID

model3 <- lme(Grain.intake.g~TRT+startbw+BW0,random =
~1|nestID3,method = "ML",data=anaSGO3,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID3, fixed = TRUE))
library(MASS)
a<-stepAIC(model3)
summary(a)
```

```
model3a <- lme(Grain.intake.g~TRT,random = ~1|nestID3,method =
"REML",data=anaSGO3,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID3, fixed = TRUE))
summary(model3a)
anova(model3a)
########POSTHOC TUKEY TEST
library(multcomp)
A11<-glht(model3a, linfct=mcp(TRT="Tukey"))
summary(A11)
B11<-cld(A11,details=TRUE,letters=c("a,b,c"))
B11
#-------------------------------WEEK 4-------------------------------
##########Subsetting data:
SGO4 <- subset(newSGO, wk == "4")
#View(SGO4)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaSGO4 <- merge(SGO4, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaSGO4)
nestID4<- anaSGO4$TRT:anaSGO4$Study.ID

model4 <- lme(Grain.intake.g~TRT+startbw+BW0,random =
~1|nestID4,method = "ML",data=anaSGO4,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID4, fixed = TRUE))
library(MASS)
a<-stepAIC(model4)
summary(a)
model4a <- lme(Grain.intake.g~TRT+startbw+BW0,random =
~1|nestID4,method = "REML",data=anaSGO4,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID4, fixed = TRUE))
```

```
summary(model4a)
anova(model4a)
########POSTHOC TUKEY TEST
library(multcomp)
A1<-glht(model4a, linfct=mcp(TRT="Tukey"))
summary(A1)
B1<-cld(A1,details=TRUE,letters=c("a,b,c"))
B1
#-----------------------------WEEK 5---------------------------------
##########Subsetting data:
SGO5 <- subset(newSGO, wk == "5")
#View(SGO5)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaSGO5 <- merge(SGO5, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaSGO5)
nestID5<- anaSGO5$TRT:anaSGO5$Study.ID

model5 <- lme(Grain.intake.g~TRT+startbw+BW0,random =
~1|nestID5,method = "ML",data=anaSGO5,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID5, fixed = TRUE))
library(MASS)
a<-stepAIC(model5)
summary(a)
model5a <- lme(Grain.intake.g~TRT,random = ~1|nestID5,method =
"REML",data=anaSGO5,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID5, fixed = TRUE))
summary(model5a)
anova(model5a)
########POSTHOC TUKEY TEST
```

```
library(multcomp)
P<-glht(model5a, linfct=mcp(TRT="Tukey"))
summary(P)
Q<-cld(P,details=TRUE,letters=c("a,b,c"))
Q
#-----------------------------WEEK 6--------------------------------
##########Subsetting data:
SGO6 <- subset(newSGO, wk == "6")
#View(SGO6)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaSGO6 <- merge(SGO6, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaSGO6)
nestID6<- anaSGO6$TRT:anaSGO6$Study.ID

model6 <- lme(Grain.intake.g~TRT+startbw+BW0,random =
~1|nestID6,method = "ML",data=anaSGO6,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID6, fixed = TRUE))
library(MASS)
a<-stepAIC(model6)
summary(a)
model6a <- lme(Grain.intake.g~startbw,random = ~1|nestID6,method =
"REML",data=anaSGO6,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID6, fixed = TRUE))
summary(model6a)
anova(model6a)

#-----------------------------WEEK 7--------------------------------
##########Subsetting data:
SGO7 <- subset(newSGO, wk == "7")
```

```
#View(SGO7)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaSGO7 <- merge(SGO7, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaSGO7)
nestID7<- anaSGO7$TRT:anaSGO7$Study.ID

model7 <- lme(Grain.intake.g~TRT+startbw+BW0,random =
~1|nestID7,method = "ML",data=anaSGO7,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID7, fixed = TRUE))
library(MASS)
a<-stepAIC(model7)
summary(a)
model7a <- lme(Grain.intake.g~startbw,random = ~1|nestID7,method =
"REML",data=anaSGO7,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID7, fixed = TRUE))
summary(model7a)
anova(model7a)

#-------------------------WEEK 8-------------------------------------
##########Subsetting data:
SGO8 <- subset(newSGO, wk == "8")
#View(SGO8)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaSGO8 <- merge(SGO8, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaSGO8)
```

```
nestID8<- anaSGO8$TRT:anaSGO8$Study.ID

model8 <- lme(Grain.intake.g~TRT+startbw+BW0,random =
~1|nestID8,method = "ML",data=anaSGO8,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID8, fixed = TRUE))
library(MASS)
a<-stepAIC(model8)
summary(a)
model8a <- lme(Grain.intake.g~startbw+BW0,random = ~1|nestID8,method =
"REML",data=anaSGO8,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID8, fixed = TRUE))
summary(model8a)
anova(model8a)

aggregate(newSGO$Grain.intake.g, by=list(newSGO$TRT, newSGO$wk),
FUN=mean,na.rm=TRUE)
aggregate(newSGO$Grain.intake.g, by=list(newSGO$TRT, newSGO$wk),
FUN=sd,na.rm=TRUE)
```

```
#WATER INTAKE

setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(lme4)

WI=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=4,
             startRow=1,
             cols=c(2:6))
#View(WI)


newWI <- WI[!with(WI,is.na("Waterintake.ml")),]
#View(newWI)
newWI$Day<- factor(newWI$Day)
newWI$wk<- factor(newWI$wk)
newWI$Study.ID <- factor(newWI$Study.ID)
newWI$TRT<- factor(newWI$TRT)
str(newWI)
nestID<- newWI$TRT:newWI$Study.ID
##----------------------------BW-data--------------------------------
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
             startRow=1,
             cols=c(2:5))
#View(bw)

newBW  <- na.omit(bw)
#View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
```

```
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)
#------------------------SUBSETTING WEEK 0 DATA-----------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
names(BWstart)[names(BWstart)=="BW"] <- "startbw"
#View(BWstart)
#-----------------------------WEEK 1---------------------------------
##########Subsetting data:
WI1 <- subset(newWI, wk == "1")
#View(WI1)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaWI1 <- merge(WI1, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaWI1)
nestID1<- anaWI1$TRT:anaWI1$Study.ID

model1 <- lme(Waterintake.ml~TRT+startbw+BW0,random =
~1|nestID1,method = "ML",data=anaWI1,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID1, fixed = TRUE))
library(MASS)
```

```
a<-stepAIC(model1)
summary(a)
model1a <- lme(Waterintake.ml~TRT,random = ~1|nestID1,method =
"REML",data=anaWI1,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID1, fixed = TRUE))
summary(model1a)
anova(model1a)
########POSTHOC TUKEY TEST
library(multcomp)
AA<-glht(model1a, linfct=mcp(TRT="Tukey"))
summary(AA)
BB<-cld(AA,details=TRUE,letters=c("a,b,c"))
BB
#----------------------------WEEK 2----------------------------------
##########Subsetting data:
WI2 <- subset(newWI, wk == "2")
#View(WI2)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaWI2 <- merge(WI2, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaWI2)
nestID2<- anaWI2$TRT:anaWI2$Study.ID

model2 <- lme(Waterintake.ml~TRT+startbw+BW0,random =
~1|nestID2,method = "ML",data=anaWI2,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID2, fixed = TRUE))
library(MASS)
a<-stepAIC(model2)
summary(a)
```

```
model2a <- lme(Waterintake.ml~TRT,random = ~1|nestID2,method =
"REML",data=anaWI2,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID2, fixed = TRUE))
summary(model2a)
anova(model2a)
########POSTHOC TUKEY TEST
library(multcomp)
A<-glht(model2a, linfct=mcp(TRT="Tukey"))
summary(A)
B<-cld(A,details=TRUE,letters=c("a,b,c"))
B
#--------------------------WEEK 3-----------------------------------
##########Subsetting data:
WI3 <- subset(newWI, wk == "3")
#View(WI3)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaWI3 <- merge(WI3, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaWI3)
nestID3<- anaWI3$TRT:anaWI3$Study.ID

model3 <- lme(Waterintake.ml~TRT+startbw+BW0,random =
~1|nestID3,method = "ML",data=anaWI3,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID3, fixed = TRUE))
library(MASS)
a<-stepAIC(model3)
summary(a)
model3a <- lme(Waterintake.ml~startbw+BW0,random = ~1|nestID3,method =
"REML",data=anaWI3,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID3, fixed = TRUE))
```

```
summary(model3a)
anova(model3a)
#------------------------------WEEK 4-------------------------------
##########Subsetting data:
WI4 <- subset(newWI, wk == "4")
#View(WI4)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaWI4 <- merge(WI4, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaWI4)
nestID4<- anaWI4$TRT:anaWI4$Study.ID

model4 <- lme(Waterintake.ml~TRT+startbw+BW0,random =
~1|nestID4,method = "ML",data=anaWI4,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID4, fixed = TRUE))
library(MASS)
a<-stepAIC(model4)
summary(a)
model4a <- lme(Waterintake.ml~TRT,random = ~1|nestID4,method =
"REML",data=anaWI4,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID4, fixed = TRUE))
summary(model4a)
anova(model4a)
########POSTHOC TUKEY TEST
library(multcomp)
C<-glht(model4a, linfct=mcp(TRT="Tukey"))
summary(C)
D<-cld(C,details=TRUE,letters=c("a,b,c"))
D
#------------------------------WEEK 5-------------------------------
```

```
##########Subsetting data:
WI5 <- subset(newWI, wk == "5")
#View(WI5)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaWI5 <- merge(WI5, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaWI5)
nestID5<- anaWI5$TRT:anaWI5$Study.ID

model5 <- lme(Waterintake.ml~TRT+startbw+BW0,random =
~1|nestID5,method = "ML",data=anaWI5,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID5, fixed = TRUE))
library(MASS)
a<-stepAIC(model5)
summary(a)
model5a <- lme(Waterintake.ml~startbw+BW0,random = ~1|nestID5,method =
"REML",data=anaWI5,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID5, fixed = TRUE))
summary(model5a)
anova(model5a)
#------------------------------WEEK 6--------------------------------
##########Subsetting data:
WI6 <- subset(newWI, wk == "6")
#View(WI6)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaWI6 <- merge(WI6, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
```

```
#View(anaWI6)
nestID6<- anaWI6$TRT:anaWI6$Study.ID

model6 <- lme(Waterintake.ml~TRT+startbw+BW0,random =
~1|nestID6,method = "ML",data=anaWI6,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID6, fixed = TRUE))
library(MASS)
a<-stepAIC(model6)
summary(a)
model6a <- lme(Waterintake.ml~startbw+BW0,random = ~1|nestID6,method =
"REML",data=anaWI6,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID6, fixed = TRUE))
summary(model6a)
anova(model6a)
#------------------------------WEEK 7--------------------------------
##########Subsetting data:
WI7 <- subset(newWI, wk == "7")
#View(WI7)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaWI7 <- merge(WI7, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaWI7)
nestID7<- anaWI7$TRT:anaWI7$Study.ID

model7 <- lme(Waterintake.ml~TRT+startbw+BW0,random =
~1|nestID7,method = "ML",data=anaWI7,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID7, fixed = TRUE))
library(MASS)
a<-stepAIC(model7)
summary(a)
```

```
model7a <- lme(Waterintake.ml~startbw+BW0,random = ~1|nestID7,method =
"REML",data=anaWI7,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID7, fixed = TRUE))
summary(model7a)
anova(model7a)
#------------------------------WEEK 8-------------------------------
##########Subsetting data:
WI8 <- subset(newWI, wk == "8")
#View(WI8)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaWI8 <- merge(WI8, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaWI8)
nestID8<- anaWI8$TRT:anaWI8$Study.ID

model8 <- lme(Waterintake.ml~TRT+startbw+BW0,random =
~1|nestID8,method = "ML",data=anaWI8,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID8, fixed = TRUE))
library(MASS)
a<-stepAIC(model8)
summary(a)
model8a <- lme(Waterintake.ml~startbw+BW0,random = ~1|nestID8,method =
"REML",data=anaWI8,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID8, fixed = TRUE))
summary(model8a)
anova(model8a)

aggregate(newWI$Waterintake.ml, by=list(newWI$TRT, newWI$wk),
FUN=mean,na.rm=TRUE)
```

```
aggregate(newWI$Waterintake.ml, by=list(newWI$TRT, newWI$wk),
FUN=sd,na.rm=TRUE)
```

```
# MILK REFUSAL
setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(lme4)

MR=read.xlsx(xlsxFile="MR_refusal.xlsx",
             sheet=3,
             startRow=1,
             cols=c(1:9))
#View(MR)

newMR <- MR[!with(MR,is.na("consumed.all.calves")),]
#View(newMR)
newMR$Day<- factor(newMR$Day)
newMR$WK<- factor(newMR$WK)
newMR$Study.ID <- factor(newMR$Study.ID)
newMR$TRT<- factor(newMR$TRT)
levels(newMR$TRT) <- list("1C"="1-CON", "2L"="2-LOW", "3H"="3-HIGH")
str(newMR)
nestID<- newMR$TRT:newMR$Study.ID
##------------------------------BW-data-----------------------------
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
             startRow=1,
             cols=c(2:5))
#View(bw)

newBW  <- na.omit(bw)
#View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
```

```
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)
#-------------------------SUBSETTING WEEK 0 DATA----------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
names(BWstart)[names(BWstart)=="BW"] <- "startbw"
#View(BWstart)
#------------------------------WEEK 1-------------------------------
##########Subsetting data:
MR1 <- subset(newMR, WK == "1")
#View(MR1)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
MR1$TRT<- factor(MR1$TRT)
MR1$Day<- factor(MR1$Day)
MR1$WK<- factor(MR1$WK)
MR1$Study.ID <- factor(MR1$Study.ID)
str(MR1)
anaMR1 <- merge(MR1, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaMR1)
nestID1<- anaMR1$TRT:anaMR1$Study.ID
```

```
model1 <- lme(consumed.all.calves~TRT+startbw+BW0,random =
~1|nestID1,method = "ML",data=anaMR1,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID1, fixed = TRUE))
library(MASS)
a<-stepAIC(model1)
summary(a)
model1a <- lme(consumed.all.calves~startbw,random = ~1|nestID1,method
= "REML",data=anaMR1,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID1, fixed = TRUE))
summary(model1a)
anova(model1a)
#---------------------------------WEEK 2-----------------------------
##########Subsetting data:
MR2 <- subset(newMR, WK == "2")
#View(MR2)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
MR2$TRT<- factor(MR2$TRT)
MR2$Day<- factor(MR2$Day)
MR2$WK<- factor(MR2$WK)
MR2$Study.ID <- factor(MR2$Study.ID)
str(MR2)
anaMR2 <- merge(MR2, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaMR2)
nestID2<- anaMR2$TRT:anaMR2$Study.ID

model2 <- lme(consumed.all.calves~TRT+startbw+BW0,random =
~1|nestID2,method = "ML",data=anaMR2,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID2, fixed = TRUE))
library(MASS)
```

```
a<-stepAIC(model2)
summary(a)
model2a <- lme(consumed.all.calves~startbw,random = ~1|nestID2,method
= "REML",data=anaMR2,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID2, fixed = TRUE))
summary(model2a)
anova(model2a)
#-------------------------------WEEK 3-------------------------------
##########Subsetting data:
MR3 <- subset(newMR, WK == "3")
#View(MR3)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
MR3$TRT<- factor(MR3$TRT)
MR3$Day<- factor(MR3$Day)
MR3$WK<- factor(MR3$WK)
MR3$Study.ID <- factor(MR3$Study.ID)
str(MR3)
anaMR3 <- merge(MR3, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaMR3)
nestID3<- anaMR3$TRT:anaMR3$Study.ID

model3 <- lme(consumed.all.calves~TRT+startbw+BW0,random =
~1|nestID3,method = "ML",data=anaMR3,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID3, fixed = TRUE))
library(MASS)
a<-stepAIC(model3)
summary(a)
```

```
model3a <- lme(consumed.all.calves~startbw+BW0,random =
~1|nestID3,method = "REML",data=anaMR3,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID3, fixed = TRUE))
summary(model3a)
anova(model3a)
#------------------------------WEEK 4--------------------------------
##########Subsetting data:
MR4 <- subset(newMR, WK == "4")
#View(MR4)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
MR4$TRT<- factor(MR4$TRT)
MR4$Day<- factor(MR4$Day)
MR4$WK<- factor(MR4$WK)
MR4$Study.ID <- factor(MR4$Study.ID)
str(MR4)
anaMR4 <- merge(MR4, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaMR4)
nestID4<- anaMR4$TRT:anaMR4$Study.ID

model4 <- lme(consumed.all.calves~TRT+startbw+BW0,random =
~1|nestID4,method = "ML",data=anaMR4,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID4, fixed = TRUE))
library(MASS)
a<-stepAIC(model4)
summary(a)
model4a <- lme(consumed.all.calves~TRT+startbw,random =
~1|nestID4,method = "REML",data=anaMR4,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID4, fixed = TRUE))
summary(model4a)
```

```
anova(model4a)
########POSTHOC TUKEY TEST
library(multcomp)
A<-glht(model4a, linfct=mcp(TRT="Tukey"))
summary(A)
B<-cld(A,details=TRUE,letters=c("a,b,c"))
B

#------------------------------WEEK 5-------------------------------
##########Subsetting data:
MR5 <- subset(newMR, WK == "5")
#View(MR5)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
MR5$TRT<- factor(MR5$TRT)
MR5$Day<- factor(MR5$Day)
MR5$WK<- factor(MR5$WK)
MR5$Study.ID <- factor(MR5$Study.ID)
str(MR5)
anaMR5 <- merge(MR5, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaMR5)
nestID5<- anaMR5$TRT:anaMR5$Study.ID

model5 <- lme(consumed.all.calves~TRT+startbw+BW0,random =
~1|nestID5,method = "ML",data=anaMR5,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID5, fixed = TRUE))
library(MASS)
a<-stepAIC(model5)
summary(a)
```

```
model5a <- lme(consumed.all.calves~TRT+BW0,random = ~1|nestID5,method
= "REML",data=anaMR5,na.action=na.omit, corr = corCAR1(form = ~ Day |
nestID5, fixed = TRUE))
summary(model5a)
anova(model5a)
########POSTHOC TUKEY TEST
library(multcomp)
C<-glht(model5a, linfct=mcp(TRT="Tukey"))
summary(C)
D<-cld(C,details=TRUE,letters=c("a,b,c"))
D

#---------------------------------WEEK 6----------------------------
##########Subsetting data:
MR6 <- subset(newMR, WK == "6")
#View(MR6)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
MR6$TRT<- factor(MR6$TRT)
MR6$Day<- factor(MR6$Day)
MR6$WK<- factor(MR6$WK)
MR6$Study.ID <- factor(MR6$Study.ID)
str(MR6)
anaMR6 <- merge(MR6, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaMR6)
nestID6<- anaMR6$TRT:anaMR6$Study.ID

model6 <- lme(consumed.all.calves~TRT+startbw+BW0,random =
~1|nestID6,method = "ML",data=anaMR6,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID6, fixed = TRUE))
```

```
library(MASS)
a<-stepAIC(model6)
summary(a)
model6a <- lme(consumed.all.calves~startbw+BW0,random =
~1|nestID6,method = "REML",data=anaMR6,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID6, fixed = TRUE))
summary(model6a)
anova(model6a)
#---------------------------------WEEK 7-----------------------------
##########Subsetting data:
MR7 <- subset(newMR, WK == "7")
#View(MR7)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
MR7$TRT<- factor(MR7$TRT)
MR7$Day<- factor(MR7$Day)
MR7$WK<- factor(MR7$WK)
MR7$Study.ID <- factor(MR7$Study.ID)
str(MR7)
anaMR7 <- merge(MR7, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaMR7)
nestID7<- anaMR7$TRT:anaMR7$Study.ID

model7 <- lme(consumed.all.calves~TRT+startbw+BW0,random =
~1|nestID7,method = "ML",data=anaMR7,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID7, fixed = TRUE))
library(MASS)
a<-stepAIC(model7)
summary(a)
```

```
model7a <- lme(consumed.all.calves~TRT+startbw+BW0,random =
~1|nestID7,method = "REML",data=anaMR7,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID7, fixed = TRUE))
summary(model7a)
anova(model7a)
########POSTHOC TUKEY TEST
library(multcomp)
E<-glht(model7a, linfct=mcp(TRT="Tukey"))
summary(E)
G<-cld(E,details=TRUE,letters=c("a,b,c"))
G
#---------------------------------WEEK 8----------------------------
##########Subsetting data:
MR8 <- subset(newMR, WK == "8")
#View(MR8)
drops <- c("WK")
BWstart1 <- BWstart[ , !(names(BWstart) %in% drops)]
#View(BWstart1)
levels(BWstart1$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
MR8$TRT<- factor(MR8$TRT)
MR8$Day<- factor(MR8$Day)
MR8$WK<- factor(MR8$WK)
MR8$Study.ID <- factor(MR8$Study.ID)
str(MR8)
anaMR8 <- merge(MR8, BWstart1, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
#View(anaMR8)
nestID8<- anaMR8$TRT:anaMR8$Study.ID

model8 <- lme(consumed.all.calves~TRT+startbw+BW0,random =
~1|nestID8,method = "ML",data=anaMR8,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID8, fixed = TRUE))
library(MASS)
```

```
a<-stepAIC(model8)
summary(a)
model8a <- lme(consumed.all.calves~startbw+BW0,random =
~1|nestID8,method = "REML",data=anaMR8,na.action=na.omit, corr =
corCAR1(form = ~ Day | nestID8, fixed = TRUE))
summary(model8a)
anova(model8a)

aggregate(newMR$consumed.all.calves, by=list(newMR$TRT, newMR$WK),
FUN=mean,na.rm=TRUE)
aggregate(newMR$consumed.all.calves, by=list(newMR$TRT, newMR$WK),
FUN=sd,na.rm=TRUE)
```

```
# HEALTH SCORE
setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
HS=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=9,
             startRow=1,
             cols=c(2:15))
HS[ HS == "." ] <- NA # make . into NA in the dataset

nnewHS<-HS[complete.cases(HS[,c(5,6,7,8)]),]
#EXCLUDE COLUMNS
drops <- c("Temp.(F)","Mark")
nnewHS$wk<- factor(nnewHS$wk)
is.factor(nnewHS$wk)
nnewHS$Study.ID <- factor(nnewHS$Study.ID)
is.factor(nnewHS$Study.ID)
nnewHS$TRT<- factor(nnewHS$TRT)
is.factor(nnewHS$TRT)
nnewHS$Appearance<-factor(nnewHS$Appearance)
is.factor(nnewHS$Appearance)
nnewHS$Fecal<-factor(nnewHS$Fecal)
is.factor(nnewHS$Fecal)
nnewHS$Respiration<-factor(nnewHS$Respiration)
is.factor(nnewHS$Respiration)
nnewHS$Nasal<-factor(nnewHS$Nasal)
is.factor(nnewHS$Nasal)
nnewHS$`Fecal.score=4(TRUE=1,.FALSE=0)`<-
factor(nnewHS$`Fecal.score=4(TRUE=1,.FALSE=0)`)
is.factor(nnewHS$`Fecal.score=4(TRUE=1,.FALSE=0)`)
nnewHS$`Fecal.score.>2.(TRUE=1,.FALSE=0)`<-
factor(nnewHS$`Fecal.score.>2.(TRUE=1,.FALSE=0)`)
is.factor(nnewHS$`Fecal.score.>2.(TRUE=1,.FALSE=0)`)
```

```
nnewHS$`Respir.Score.>2(TRUE=1,.FALSE=0)`<-
factor(nnewHS$`Respir.Score.>2(TRUE=1,.FALSE=0)`)
is.factor(nnewHS$`Respir.Score.>2(TRUE=1,.FALSE=0)`)
nnewHS$`Nasal.Score.>2(TRUE=1,.FALSE=0)`<-
factor(nnewHS$`Nasal.Score.>2(TRUE=1,.FALSE=0)`)
is.factor(nnewHS$`Nasal.Score.>2(TRUE=1,.FALSE=0)`)

#Exclude columns
ddrops <-
c("Day","Fecal.score=4(TRUE=1,.FALSE=0)","Fecal.score.>2.(TRUE=1,.FALS
E=0)","Respir.Score.>2(TRUE=1,.FALSE=0)","Nasal.Score.>2(TRUE=1,.FALSE
=0)")
nnnewHS<-nnewHS[ , !(names(nnewHS) %in% ddrops)]
View(nnnewHS)

#####Multinomial logit model
https://dss.princeton.edu/training/LogitR101.pdf and
http://data.princeton.edu/wws509/r/c6s2.html
mydata=nnnewHS
library(foreign)
library(nnet)
library(stargazer)
str(mydata)
######NASAL########
mydata$nasal <- relevel(mydata$Nasal, ref="2")
multi1 = multinom(Nasal ~ TRT*wk,
data=mydata,random=~1|Study.ID,na.action = na.omit)
summary(multi1)
stargazer(multi1, type="text", out="multi1.txt")

## risk ratios
multi1.rrr = exp(coef(multi1))
```

```
stargazer(multi1, type="text",
out="multi1risk.txt",coef=list(multi1.rrr), p.auto=FALSE)

######APPEARRANCE#####
multi3 = multinom(Appearance ~ TRT*wk,
data=mydata,random=~1|Study.ID,na.action = na.omit)
summary(multi3)
stargazer(multi3, type="text", out="multi3.txt")

## risk ratios
multi3.rrr = exp(coef(multi3))
stargazer(multi3, type="text",
out="multi3risk.txt",coef=list(multi3.rrr), p.auto=FALSE)

######RESPIRATION######
multi4 = multinom(Respiration ~ TRT*wk,
data=mydata,random=~1|Study.ID,na.action = na.omit)
summary(multi4)
stargazer(multi4, type="text", out="multi4.txt")

## risk ratios
multi4.rrr = exp(coef(multi4))
stargazer(multi4, type="text",
out="multi4risk.txt",coef=list(multi4.rrr), p.auto=FALSE)
```

```
# GIT weight
setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(multcomp)

GIT=read.xlsx(xlsxFile="rumen_pH_GIT_kg.xlsx",
              sheet=1,
              startRow=1,
              cols=c(1:4,6))
View(GIT)

is.numeric(GIT$"GIT.kg")
GIT$die.day<- factor(GIT$die.day)
is.factor(GIT$die.day)
GIT$order.die<- factor(GIT$order.die)
is.factor(GIT$order.die)
GIT$Study.ID <- factor(GIT$Study.ID)
is.factor(GIT$Study.ID)
GIT$TRT<- factor(GIT$TRT)
is.factor(GIT$TRT)

nestID<- GIT$TRT:GIT$Study.ID
#--------------------------------BW WK 8-----------------------------
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
              sheet=2,
              startRow=1,
              cols=c(2:5))
View(bw)

newBW  <- na.omit(bw)
View(newBW)
is.numeric(newBW$BW)
```

```
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)

BW8 <- subset(newBW, WK == "8")
View(BW8)
drops <- c("WK")
BWWK8 <- BW8[ , !(names(BW8) %in% drops)]
names(BWWK8)[names(BWWK8) == 'BW'] <- 'BW8'
View(BWWK8)
levels(BWWK8$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
GITBW1 <- merge(BWWK8, GIT, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(GITBW1)
#----------------------SUBSETTING WEEK 0 DATA-----------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
levels(BWstart$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
#View(BWstart)

GITBW <- merge(GITBW1, BWstart, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
write.table(GITBW, "GITBW.txt", sep="\t", quote=F)
str(GITBW)
```

```
head(GITBW)
nestID <- GITBW$TRT:GITBW$Study.ID
#--------------------------------analysis-----------------------------
model1 <- lme(GIT.kg~TRT+order.die+die.day+BW+BW0+BW8,random =
~1|nestID,method = "ML",data=GITBW,na.action=na.omit)
library(MASS)
a<-stepAIC(model1)
model1a<-lme(GIT.kg~TRT+order.die+BW+BW0+BW8,random = ~1|nestID,method
= "REML",data=GITBW,na.action=na.omit)
anova(model1a)
########POSTHOC TUKEY TEST
A<-glht(model1a, linfct=mcp(TRT="Tukey"))
summary(A)
B<-cld(A,details=TRUE,letters=c("a,b,c"))
B

aggregate(GITBW$GIT.kg, by=list(GITBW$TRT), FUN=mean,na.rm=TRUE)
aggregate(GITBW$GIT.kg, by=list(GITBW$TRT), FUN=sd,na.rm=TRUE)
```

```
#pH rumen
setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(lme4)

ph=read.xlsx(xlsxFile="rumen_pH_GIT_kg.xlsx",
              sheet=1,
              startRow=1,
              cols=c(1:5))
View(ph)

is.numeric(ph$"PH")
ph$die.day<- factor(ph$die.day)
is.factor(ph$die.day)
ph$order.die<- factor(ph$order.die)
is.factor(ph$order.die)
ph$Study.ID <- factor(ph$Study.ID)
is.factor(ph$Study.ID)
ph$TRT<- factor(ph$TRT)
is.factor(ph$TRT)

nestID<- ph$TRT:ph$Study.ID
#------------------------------BW WK 8--------------------------------
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
             startRow=1,
             cols=c(2:5))
View(bw)

newBW  <- na.omit(bw)
View(newBW)
is.numeric(newBW$BW)
```

```
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)

BW8 <- subset(newBW, WK == "8")
View(BW8)
drops <- c("WK")
BWWK8 <- BW8[ , !(names(BW8) %in% drops)]
View(BWWK8)
levels(BWWK8$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
phBW <- merge(BWWK8, ph, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(phBW)
#-------------------------------analysis-----------------------------
model1 <- lme(PH~TRT+order.die+die.day+BW,random = ~1|nestID,method =
"ML",data=phBW,na.action=na.omit)
library(MASS)
a<-stepAIC(model1)
summary(a)
model1a<-lme(PH~BW,random = ~1|nestID,method =
"REML",data=phBW,na.action=na.omit)
anova(model1a)


aggregate(phBW$PH, by=list(phBW$TRT), FUN=mean,na.rm=TRUE)
aggregate(phBW$PH, by=list(phBW$TRT), FUN=sd,na.rm=TRUE)
```

```
# RUMEN MORPH
setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(multcomp)

rumlen=read.xlsx(xlsxFile="rumen_hist.xlsx",
                 sheet=1,
                 startRow=3,
                 cols=c(1:8))
View(rumlen)

is.data.frame(rumlen)
str(rumlen)
rumlen$Obs <- factor(rumlen$Obs)
rumlen$slide <- factor(rumlen$slide)
rumlen$calf <- factor(rumlen$calf)
rumlen$trt <- factor(rumlen$trt)
rumlen$count <- factor(rumlen$count)
names(rumlen)[names(rumlen) == 'calf'] <- 'Study.ID'
names(rumlen)[names(rumlen) == 'trt'] <- 'TRT'
str(rumlen)


#--------------------------BW WK 8----------------------------------
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
             startRow=1,
             cols=c(2:5))
View(bw)

newBW  <- na.omit(bw)
View(newBW)
```

```
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)

BW8 <- subset(newBW, WK == "8")
View(BW8)
drops <- c("WK")
BWWK8 <- BW8[ , !(names(BW8) %in% drops)]
names(BWWK8)[names(BWWK8) == 'BW'] <- 'BW8'
View(BWWK8)
levels(BWWK8$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anarumlen1 <- merge(BWWK8, rumlen, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anarumlen1)
anarumlen1 <- anarumlen1[ ! anarumlen1$Study.ID %in% c(22), ]

#-------------------------SUBSETTING WEEK 0 DATA----------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
levels(BWstart$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
#View(BWstart)
```

```
anarumlen <- merge(anarumlen1, BWstart, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
write.table(anarumlen, "anarumlen.txt", sep="\t", quote=F)
str(anarumlen)
head(anarumlen)
nestID <- anarumlen$TRT:anarumlen$Study.ID
#-------------------------analysis - Length-------------------------
model1 <- lme(length~TRT+BW+BW0+BW8,random = ~1|nestID,method =
"ML",data=anarumlen,na.action=na.omit)
library(MASS)
a<-stepAIC(model1)
model1a<-lme(length~TRT+BW+BW0+BW8,random = ~1|nestID,method =
"REML",data=anarumlen,na.action=na.omit)
anova(model1a)
########POSTHOC TUKEY TEST
A<-glht(model1a, linfct=mcp(TRT="Tukey"))
summary(A)
B<-cld(A,details=TRUE,letters=c("a,b,c"))
B
#---------------------------analysis - Width-------------------------
model2 <- lme(width~TRT+BW+BW0+BW8,random = ~1|nestID,method =
"ML",data=anarumlen,na.action=na.omit)
library(MASS)
b<-stepAIC(model2)
model2a<-lme(width~BW8,random = ~1|nestID,method =
"REML",data=anarumlen,na.action=na.omit)
anova(model2a)

#-------------------------analysis - Ratio-------------------------
model3 <- lme(ratio~TRT+BW+BW0+BW8,random = ~1|nestID,method =
"ML",data=anarumlen,na.action=na.omit)
library(MASS)
c<-stepAIC(model3)
```

```
model3a<-lme(ratio~TRT+BW+BW0+BW8,random = ~1|nestID,method =
"REML",data=anarumlen,na.action=na.omit)
anova(model3a)
########POSTHOC TUKEY TEST
C<-glht(model3a, linfct=mcp(TRT="Tukey"))
summary(C)
D<-cld(C,details=TRUE,letters=c("a,b,c"))
D

aggregate(anarumlen$length, by=list(anarumlen$TRT, anarumlen$WK),
FUN=mean,na.rm=TRUE)
aggregate(anarumlen$length, by=list(anarumlen$TRT, anarumlen$WK),
FUN=sd,na.rm=TRUE)
aggregate(anarumlen$width, by=list(anarumlen$TRT, anarumlen$WK),
FUN=mean,na.rm=TRUE)
aggregate(anarumlen$width, by=list(anarumlen$TRT, anarumlen$WK),
FUN=sd,na.rm=TRUE)
aggregate(anarumlen$ratio, by=list(anarumlen$TRT, anarumlen$WK),
FUN=mean,na.rm=TRUE)
aggregate(anarumlen$ratio, by=list(anarumlen$TRT, anarumlen$WK),
FUN=sd,na.rm=TRUE)
```

```
# JEJUNUM HISTO - villus heigth

setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)

jejuvil=read.xlsx(xlsxFile="jejunum_hist.xlsx",
                  sheet=1,
                  startRow=3,
                  cols=c(1:7))
View(jejuvil)

is.data.frame(jejuvil)
str(jejuvil)
jejuvil$Obs <- factor(jejuvil$Obs)
jejuvil$item <- factor(jejuvil$item)
jejuvil$calf <- factor(jejuvil$calf)
jejuvil$trt <- factor(jejuvil$trt)
names(jejuvil)[names(jejuvil) == 'calf'] <- 'Study.ID'
names(jejuvil)[names(jejuvil) == 'trt'] <- 'TRT'
str(jejuvil)


#--------------------------BW WK 8----------------------------------
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
             startRow=1,
             cols=c(2:5))
View(bw)

newBW  <- na.omit(bw)
View(newBW)
is.numeric(newBW$BW)
```

```
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)

BW8 <- subset(newBW, WK == "8")
View(BW8)
drops <- c("WK")
BWWK8 <- BW8[ , !(names(BW8) %in% drops)]
names(BWWK8)[names(BWWK8) == 'BW'] <- 'BW8'
View(BWWK8)
levels(BWWK8$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anajejuVH1 <- merge(BWWK8, jejuvil, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anajejuVH1)
anajejuVH1 <- anajejuVH1[ ! anajejuVH1$Study.ID %in% c(22), ]

#----------------------SUBSETTING WEEK 0 DATA-------------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
levels(BWstart$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
#View(BWstart)

anajejuVH <- merge(anajejuVH1, BWstart, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
```

```
write.table(anajejuVH, "anajejuVH.txt", sep="\t", quote=F)
str(anajejuVH)
head(anajejuVH)
nestID <- anajejuVH$TRT:anajejuVH$Study.ID
#---------------------------analysis----------------------------------
model1 <- lme(VH~TRT+BW+BW0+BW8,random = ~1|nestID,method =
"ML",data=anajejuVH,na.action=na.omit)
library(MASS)
a<-stepAIC(model1)
model1a<-lme(VH~BW+BW0,random = ~1|nestID,method =
"REML",data=anajejuVH,na.action=na.omit)
anova(model1a)

aggregate(anajejuVH$VH, by=list(anajejuVH$TRT, anajejuVH$WK),
FUN=mean,na.rm=TRUE)
aggregate(anajejuVH$VH, by=list(anajejuVH$TRT, anajejuVH$WK),
FUN=sd,na.rm=TRUE)
```

```
# JEJUNUM HISTO - crypt depth
# least square means analyse

setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(lme4)

jejucrypt=read.xlsx(xlsxFile="jejunum_hist.xlsx",
                sheet=1,
                startRow=3,
                cols=c(1:7))
View(jejucrypt)

is.data.frame(jejucrypt)
str(jejucrypt)
jejucrypt$Obs <- factor(jejucrypt$Obs)
jejucrypt$item <- factor(jejucrypt$item)
jejucrypt$calf <- factor(jejucrypt$calf)
jejucrypt$trt <- factor(jejucrypt$trt)
names(jejucrypt)[names(jejucrypt) == 'calf'] <- 'Study.ID'
names(jejucrypt)[names(jejucrypt) == 'trt'] <- 'TRT'
str(jejucrypt)


#----------------------------BW WK 8---------------------------------
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
             startRow=1,
             cols=c(2:5))
View(bw)

newBW  <- na.omit(bw)
```

```
View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)

BW8 <- subset(newBW, WK == "8")
View(BW8)
drops <- c("WK")
BWWK8 <- BW8[ , !(names(BW8) %in% drops)]
names(BWWK8)[names(BWWK8) == 'BW'] <- 'BW8'
View(BWWK8)
levels(BWWK8$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anajejuCD1 <- merge(BWWK8, jejucrypt, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anajejuCD1)
anajejuCD1 <- anajejuCD1[ ! anajejuCD1$Study.ID %in% c(22), ]
nestID <- anajejuCD1$TRT:anajejuCD1$Study.ID
#------------------------SUBSETTING WEEK 0 DATA----------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
levels(BWstart$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
#View(BWstart)
```

```
anajejuCD <- merge(anajejuCD1, BWstart, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
write.table(anajejuCD, "anajejuCD.txt", sep="\t", quote=F)
str(anajejuCD)
head(anajejuCD)
nestID <- anajejuCD$TRT:anajejuCD$Study.ID
#-------------------------analysis-------------------------------------
model1 <- lme(CD~TRT+BW+BW0+BW8,random = ~1|nestID,method =
"ML",data=anajejuCD,na.action=na.omit)
library(MASS)
a<-stepAIC(model1)
model1a<-lme(CD~BW0,random = ~1|nestID,method =
"REML",data=anajejuCD,na.action=na.omit)
anova(model1a)

aggregate(anajejuCD$CD, by=list(anajejuCD$TRT, anajejuCD$WK),
FUN=mean,na.rm=TRUE)
aggregate(anajejuCD$CD, by=list(anajejuCD$TRT, anajejuCD$WK),
FUN=sd,na.rm=TRUE)
```

```
# JEJUNUM HISTO - villus to crypt ratio

setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(multcomp)

jejurat=read.xlsx(xlsxFile="jejunum_hist.xlsx",
                  sheet=1,
                  startRow=3,
                  cols=c(1:7))
View(jejurat)

is.data.frame(jejurat)
str(jejurat)
jejurat$Obs <- factor(jejurat$Obs)
jejurat$item <- factor(jejurat$item)
jejurat$calf <- factor(jejurat$calf)
jejurat$trt <- factor(jejurat$trt)
names(jejurat)[names(jejurat) == 'calf'] <- 'Study.ID'
names(jejurat)[names(jejurat) == 'trt'] <- 'TRT'
str(jejurat)


#-----------------------------BW WK 8--------------------------------
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
             startRow=1,
             cols=c(2:5))
View(bw)

newBW  <- na.omit(bw)
View(newBW)
```

```
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)

BW8 <- subset(newBW, WK == "8")
View(BW8)
drops <- c("WK")
BWWK8 <- BW8[ , !(names(BW8) %in% drops)]
names(BWWK8)[names(BWWK8) == 'BW'] <- 'BW8'
View(BWWK8)
levels(BWWK8$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anajejuR1 <- merge(BWWK8, jejurat, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anajejuR1)
anajejuR1 <- anajejuR1[ ! anajejuR1$Study.ID %in% c(22), ]

#----------------------SUBSETTING WEEK 0 DATA-------------------------
BWstart <- subset(newBW, WK == "0")
#View(BWstart)

BWstart["BW0"] <- NA # That creates the new column named "BW0" filled
with "NA"
BWstart$BW0 <- BWstart$BW^2  # As an example, the new column receive
#View(BWstart)
BWstart$BW0
levels(BWstart$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
#View(BWstart)
```

```
anajejuR <- merge(anajejuR1, BWstart, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
write.table(anajejuR, "anajejuR.txt", sep="\t", quote=F)
str(anajejuR)
head(anajejuR)
nestID <- anajejuR$TRT:anajejuR$Study.ID
#-----------------------------analysis---------------------------------
model1 <- lme(ratio~TRT+BW+BW0+BW8,random = ~1|nestID,method =
"ML",data=anajejuR,na.action=na.omit)
library(MASS)
a<-stepAIC(model1)
model1a<-lme(ratio~TRT+BW,random = ~1|nestID,method =
"REML",data=anajejuR,na.action=na.omit)
anova(model1a)
########POSTHOC TUKEY TEST
A<-glht(model1a, linfct=mcp(TRT="Tukey"))
summary(A)
B<-cld(A,details=TRUE,letters=c("a,b,c"))
B

aggregate(anajejuR$ratio, by=list(anajejuR$TRT, anajejuR$WK),
FUN=mean,na.rm=TRUE)
aggregate(anajejuR$ratio, by=list(anajejuR$TRT, anajejuR$WK),
FUN=sd,na.rm=TRUE)
```

```
# JEJUNUM HISTO - HRP flux

setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(lme4)

HRP=read.xlsx(xlsxFile="jejunum_flux.xlsx",
                    sheet=1,
                    startRow=3,
                    cols=c(1:7))
View(HRP)

is.data.frame(HRP)
str(HRP)
HRP$Obs <- factor(HRP$Obs)
HRP$tissue <- factor(HRP$tissue)
HRP$calf <- factor(HRP$calf)
HRP$trt <- factor(HRP$trt)
HRP$time <- factor(HRP$time)
HRP$rep <- factor(HRP$rep)
names(HRP)[names(HRP) == 'calf'] <- 'Study.ID'
names(HRP)[names(HRP) == 'trt'] <- 'TRT'
str(HRP)


#------------------------------BW WK 8------------------------------
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
             startRow=1,
             cols=c(2:5))
View(bw)
```

```
newBW  <- na.omit(bw)
View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)

BW8 <- subset(newBW, WK == "8")
View(BW8)
drops <- c("WK")
BWWK8 <- BW8[ , !(names(BW8) %in% drops)]
names(BWWK8)[names(BWWK8) == 'BW'] <- 'BW8'
View(BWWK8)
levels(BWWK8$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
levels(HRP$TRT) <- list("1C"="c", "2L"="l", "3H"="h")
anaHRP1 <- merge(BWWK8, HRP, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaHRP1)
anaHRP1 <- anaHRP1[!is.na(anaHRP1$flux),]
nestID <- anaHRP1$TRT:anaHRP1$Study.ID
#--------------------------analysis---------------------------------
model1 <- lme(flux~(TRT*time)+BW8,random = ~1|nestID,method =
"ML",data=anaHRP1,na.action=na.omit)
library(MASS)
a<-stepAIC(model1)
model1a<-lme(flux~BW8,random = ~1|nestID,method =
"REML",data=anaHRP1,na.action=na.omit)
anova(model1a)

aggregate(anaHRP1$flux, by=list(anaHRP1$TRT), FUN=mean,na.rm=TRUE)
```

```
aggregate(anaHRP1$flux, by=list(anaHRP1$TRT), FUN=sd,na.rm=TRUE)
```

```
# JEJUNUM HISTO - FD4 flux
# least square means analyse

setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)
library(lme4)

FD4=read.xlsx(xlsxFile="jejunum_flux.xlsx",
              sheet=2,
              startRow=3,
              cols=c(1:7))
View(FD4)

is.data.frame(FD4)
str(FD4)
FD4$Obs <- factor(FD4$Obs)
FD4$tissue <- factor(FD4$tissue)
FD4$calf <- factor(FD4$calf)
FD4$trt <- factor(FD4$trt)
FD4$time <- factor(FD4$time)
FD4$rep <- factor(FD4$rep)
names(FD4)[names(FD4) == 'calf'] <- 'Study.ID'
names(FD4)[names(FD4) == 'trt'] <- 'TRT'
str(FD4)


#--------------------------------BW WK 8-----------------------------
bw=read.xlsx(xlsxFile="production_data_april.xlsx",
             sheet=2,
             startRow=1,
             cols=c(2:5))
View(bw)
```

```
newBW  <- na.omit(bw)
View(newBW)
is.numeric(newBW$BW)
newBW$WK<- factor(newBW$WK)
is.factor(newBW$WK)
newBW$Study.ID <- factor(newBW$Study.ID)
is.factor(newBW$Study.ID)
newBW$TRT<- factor(newBW$TRT)
is.factor(newBW$TRT)

BW8 <- subset(newBW, WK == "8")
View(BW8)
drops <- c("WK")
BWWK8 <- BW8[ , !(names(BW8) %in% drops)]
names(BWWK8)[names(BWWK8) == 'BW'] <- 'BW8'
View(BWWK8)
levels(BWWK8$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
levels(FD4$TRT) <- list("1C"="C", "2L"="L", "3H"="H")
anaFD4 <- merge(BWWK8, FD4, by.x=c("Study.ID","TRT"),
by.y=c("Study.ID","TRT"),all=TRUE,all.x=TRUE)
View(anaFD4)
anaFD4 <- anaFD4[!is.na(anaFD4$flux),]
nestID <- anaFD4$TRT:anaFD4$Study.ID
#------------------------------analysis--------------------------------
model1 <- lme(flux~(TRT*time)+BW8,random = ~1|nestID,method =
"ML",data=anaFD4,na.action=na.omit)
library(MASS)
a<-stepAIC(model1)
model1a<-lme(flux~time,random = ~1|nestID,method =
"REML",data=anaFD4,na.action=na.omit)
anova(model1a)
```

```
aggregate(anaFD4$flux, by=list(anaFD4$TRT), FUN=mean,na.rm=TRUE)
aggregate(anaFD4$flux, by=list(anaFD4$TRT), FUN=sd,na.rm=TRUE)
```

```
#-----------------------------GENEEXPRESSION-------------------------
setwd("~/Desktop/louisehilligsoe/Documents/KU/Speciale/STATs")
library(openxlsx)
library(nlme)

geneex=read.xlsx(xlsxFile="Gene_expression_april.xlsx",
                 sheet=6,
                 startRow=1,
                 cols = c(1:6))
geneex[is.na(geneex)]=0
#View(geneex)

geneex$Rep<- factor(geneex$Rep)
geneex$Calf.ID <- factor(geneex$Calf.ID)
geneex$Trt<- factor(geneex$Trt)
str(geneex)
nestID1<- geneex$Trt:geneex$Calf.ID
#---------------------------ANALYSIS CLDN1---------------------------
model1 <- lme(CLDN1~Trt,random = ~1|nestID1,method =
"ML",data=geneex,na.action=na.omit)
library(MASS)
a<-stepAIC(model1)
summary(a)
model1a<-lme(CLDN1~Trt,random = ~1|nestID1,method =
"REML",data=geneex,na.action=na.omit)
summary(model1a)
anova(model1a)
########POSTHOC TUKEY TEST
library(multcomp)
A<-glht(model1a, linfct=mcp(Trt="Tukey"))
summary(A)
B<-cld(A,details=TRUE,letters=c("a,b,c"))
B
```

```
#-----------------------------ANALYSIS TIJ1-------------------------
model2 <- lme(TIJ1~Trt,random = ~1|nestID1,method =
"ML",data=geneex,na.action=na.omit)
library(MASS)
b<-stepAIC(model2)
summary(b)
model2a<-lme(TIJ1~Trt,random = ~1|nestID1,method =
"REML",data=geneex,na.action=na.omit)
summary(model2a)
anova(model2a)
########POSTHOC TUKEY TEST
C<-glht(model2a, linfct=mcp(Trt="Tukey"))
summary(C)
D<-cld(C,details=TRUE,letters=c("a,b,c"))
D
#----------------------------ANALYSIS OCLN----------------------------
model3 <- lme(OCLN~Trt,random = ~1|nestID1,method =
"ML",data=geneex,na.action=na.omit)
library(MASS)
c<-stepAIC(model3)
summary(c)
model3a<-lme(OCLN~Trt,random = ~1|nestID1,method =
"REML",data=geneex,na.action=na.omit)
summary(model3a)
anova(model3a)
########POSTHOC TUKEY TEST
E<-glht(model3a, linfct=mcp(Trt="Tukey"))
summary(E)
G<-cld(E,details=TRUE,letters=c("a,b,c"))
G
```