The effect of milk replacers containing fermented plant protein and a higher carbohydrate content on the growth performance and profitability of Holstein bull calves.

by
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Declaration

I, Elsa Ena Kriel, declare that the Master's Degree research dissertation or interrelated, publishable manuscripts/published articles, or coursework Master's Degree mini-dissertation that I herewith submit for the Master's Degree qualification at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

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Date: 06/12/2024

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Abstract

The objective of this study was to evaluate the biological and economic viability of alternative milk replacers for Holstein bull calves, focusing on high-carbohydrate and fermented plant protein-based formulations. The primary goals were to assess the impact of these alternatives on calf growth, health, and profitability by identify the most cost-effective options for farmers.

Chapter 3 investigate the effects of fermented plant protein (FP) and increased carbohydrate (HC) levels in milk replacers on calf growth performance. Four different milk replacers were formulated: a conventional replacer (A), one with 20% fermented plant protein (B), a high-carbohydrate replacer (C), and a high-carbohydrate replacer with 20% fermented protein (D). These milk replacers were tested in a 77-day trial (TP, total phase) involving 32 Holstein bull calves, which were randomly assigned to four treatments (n = 8). The trial was divided into two phases: Phase 1 (P1, days 0–63), where milk replacers were fed, and Phase 2 (P2, a two-week post-weaning period), designed to evaluate weaning shock. Milk replacer intake was controlled, while starter meal intake was offered *ad libitum*. Growth performance metrics such as weight gain, dry matter intake (DMI), average daily gain (ADG), DMI as a percentage of body weight (DMI/BW%), and feed conversion ratio (FCR) were analysed using a factorial ANOVA. An interaction was observed for TP FCR ($P = 0.036$). Except for controlled milk replacer intake and P2 DMI/BW% and P2 FCR, milk replacers containing FP generally resulted in poorer performance ($P < 0.05$) compared to milk replacers containing standard protein (SP). The lower performance was attributed to the higher levels of trypsin inhibitors present in the FP, which negatively impacted calf growth. High-carbohydrate milk replacers promoted greater starter meal intake during all phases ($P < 0.05$) and is recommended. Fermented protein should only be considered when the fermentation process is improved to reduce trypsin inhibitor levels to below 4 mg/g of protein.

Chapter 4 shifts focus to the financial analysis, comparing the cost-effectiveness of the different milk replacers (A, B, C and D). Financial parameters such as total cost, average daily cost (ADC), cost per weight gain (Cost/WD), and income from the sale of calves were assessed. In terms of overall cost and ADC, treatment D was the most economical, with a total feeding cost of R2739.40 per calf for TP. However, when factoring in feed conversion ratio (FCR), treatment C was the most cost-effective, with the highest income and lowest cost per unit weight gain (R58.83), although not significantly different when compared to Treatments A and D.

Chapter 5 extends the financial analysis by exploring the use of girth circumference weight estimation tapes as an alternative to expensive electronic scales. This method is valuable for calf management, as weight is crucial for feed management and medication protocols. The study found that while the specific tape used in the trial overestimated calf weights, it holds potential as a low-cost alternative, with further refinement needed. The data collected can contribute to the development of a larger database to improve future weight prediction tools.

In conclusion, this study demonstrates that high-carbohydrate milk replacers are a viable and cost-effective alternative to conventional formulations, offering potential cost savings without sacrificing calf growth performance. While fermented plant protein showed promise as a cost-effective protein source, its effectiveness in this trial was reduced by the presence of trypsin inhibitors, which could be addressed through improved processing methods. Additionally, Chapter 5's exploration of girth circumference measurement as a cost-effective weight estimation tool further supports the economic viability of alternative approaches to calf rearing. The findings highlight the need for ongoing research to optimize these alternatives and improve profitability in the dairy industry.

Opsomming

Die doel van die studie was om die biologiese en ekonomiese lewensvatbaarheid van alternatiewe melkvervangers vir Holstein bulkalwers te evalueer, met die fokus op formulasies met ‘n hoër koolhidraat en gefermenteerde plantproteïen insluitings. Die primêre doelwitte was om die impak van hierdie alternatiewe op kalfgroei, gesondheid en winsgewendheid te evalueer.

In **Hoofstuk 3** is die effek van gefermenteerde plant proteïen (FP) en verhoogde koolhidraat (HC) insluitings vlakke deur vier melkvervangers vir kalwers ondersoek. Die vier melkvervangers was 'n standaard melkvervanger (A), een wat 20% gefermenteerde plantproteïen bevat het (B), 'n hoë-koolhidraat melkvervanger (C) en 'n hoë-koolhidraat melkvervanger wat 20% gefermenteerde proteïen bevat het (D). Hierdie melkvervangers is getoets in 'n 77-dae proef (TP, totale fase) met 32 Holstein bulkalwers, wat ewekansig aan vier behandelings (n = 8) toegeken is. Die proef is in twee fases verdeel: Fase 1 (P1, dae 0–63), waar melkvervangers gevoer is, en Fase 2 (P2, 'n twee weke na-speen tydperk), wat gebruik is om speenskok te evalueer. Melkvervanger-inname was beheer, terwyl die aanvangsmeel *ad libitum* beskikbaar was. Groeiprestasie-maatstawwe soos gewigstoename, droëmateriaal-inname (DMI), gemiddelde daaglikse toename (GDT), DMI as 'n persentasie van liggaamsgewig (DMI/BW%) en voeromsettingsverhouding (FCR) is met behulp van 'n faktoriale ANOVA ontleed. 'n Interaksie is waargeneem vir TP FCR ($P = 0.036$). Behalwe vir beheerde melkvervanger-inname en P2 DMI/BW% en P2 FCR, het melkvervangers wat FP bevat, oor die algemeen swakker prestasie ($P < 0.05$) tot gevolg gehad in vergelyking met standaard proteïen (SP) melkvervangers. Die laer prestasie kan toegeskryf word aan die hoër vlakke van tripsien-inhibeerders teenwoordig in die FP, wat kalfgroei onderdruk het. Hoë-koolhidraat melkvervangers het inname van die aanvangsmeel gedurende alle fases bevorder ($P < 0,05$) en word aanbeveel. Gefermenteerde proteïen moet slegs oorweeg word wanneer die fermentasieproses verbeter word om tripsieninhibeerdervlakke tot onder 4 mg/g proteïen te verminder.

In **Hoofstuk 4** het die fokus na die finansiële aspekte verskuif, waar die koste-doeltreffendheid van hierdie melkvervangers (A, B, C en D) geëvalueer is. Finansiële parameters soos totale koste, gemiddelde daaglikse koste (ADC), koste per gewigstoename (Cost/WD), en inkomste uit die verkoop van kalwers is ealueer. Wat algehele koste en ADC betref, het Behandeling D die beste presteer, met 'n totale voerkoste van R2739.40 per kalf vir TP. Wanneer voeromsettingsdoeltreffenheid (FCR) inag geneem word, was Behandeling C egter die mees kostedoeltreffende opsie, met die hoogste inkomste en die laagste koste per eenheid gewigstoename (R58,83), alhoewel daar geen beduidende verskil was in vergelyking met Behandelings A en D nie.

Hoofstuk 5 het die finansiële analise uitgebrei deur die gebruik van borsomtrekmaatband as 'n alternatief vir duur elektroniese skale te ondersoek. Hierdie hoofstuk het ten doel gehad om hierdie

laekoste-metode verder te verifieer as metode om die gewig van kalwers te beraam, aangesien gewig 'n noodsaaklike bestuurshulpmiddel is wat gebruik word tydens voerbestuur en medikasieprotokolle. Die studie het bevind dat hoewel die spesifieke maatband wat in die proef gebruik is, die gewig van kalwers oorskat het, die data wat ingewin is kan bydra tot die ontwikkeling van 'n groter databasis wat gebruik kan word om meer akkurate voorspellingsgereedskap in die toekoms te ontwikkel.

Ten slotte het die studie getoon dat hoë-koolhidraat melkvervangers 'n lewensvatbare, koste-effektiewe alternatief vir konvensionele melkvervangers is. Dit het die potensiaal om koste besparings te bied sonder om kalfgroei te benadeel. Terwyl gefermenteerde plantproteïen belofte toon as 'n koste-effektiewe proteïenbron, was die prestasie in hierdie proef onderdruk deur die teenwoordigheid van tripsien-inhibeerders. Dit kan aangespreek word deur beter verwerkingsmetodes te ontwikkel om die tripsien-inhibeerders te de-aktiveer. Die borsomvangmaatband kan as 'n laekoste manier om gewig te beraam bydra tot die ekonomiese lewensvatbaarheid van kalfgrootmakers. Die bevindings beklemtoon die behoefte aan voortgesette navorsing om hierdie alternatiewe te optimaliseer vir verbeterde winsgewendheid in kalfproduksie.

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Preface

This thesis is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the South African Journal of Animal Science to which Chapter 2, 3, 4 and 5 will be submitted for publication.

Chapter 1 **General Introduction**

General introduction to the milk replacers industry (international and domestic), project motivation, aims and objective.

Chapter 2 **Literature review**

The pre-ruminant digestive enzymes and the influnces of milk replacers, procesing methods and weaning shock.

Chapter 3 **Article 1 - Research results**

The effect of milk replacers containing fermented plant protein and high carbohydrate content on the growth performance of Holstein bull calves.

Chapter 4 **Article 2 - Research results**

The effect of using different protein sources and a high level of carbohydrates in milk replacers on the profitability of rearing Holstein bull calves.

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List of Abbreviations

General:

US	Stellenbosch University
AS	Faculty of AgriSciences
UFS	University of the Free State

Literature review:

Δ	Energy difference (Delta)
$\Delta G^{\ddagger}_{cat}$	Catalysed transition state free energy
$\Delta G^{\ddagger}_{uncat}$	Uncatalysed transition state free energy
ΔG_{RXN}	Overall free energy releases during reaction
‡	Transition state
$\ddagger_{cat}$	Catalysed reaction transition state
$\ddagger_{uncat}$	Uncatalysed reaction transition state
C	Carbon
C1	First carbon
C4:0	Butyric acid
CFA	Cis fatty acids
CH_4	Methane
CO_2	Carbon dioxide
COOH	Carboxylic acid (Carboxyl group)
DIAAS	Digestible Indispensable Amino Acid Score
DM	Dry matter
EP	Enzyme-product
ES	Enzyme-substrate
FA	Fatty acids
G	Gibbs energy (Free energy)
H	Hydrogen
HCl	Hydrochloric acid (Gastric acid)
IgG	Immunoglobulin G
k_-	Backward reaction kinetic energy
K	Potassium
k_+	Forward reaction kinetic energy
LCFA	Long-chain fatty acids
Maltase-GA	Maltase-glucoamylase

MCFA	Medium-chain fatty acids
MUFA	Mono-unsaturated fatty acids
n-3	Omega-3 fatty acids
n-6	Omega-6 fatty acids
n-9	Omega-9 fatty acids
Na	Sodium
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NH_2	Amino group
NH_3	Ammonia
NH_4OH	Ammonium hydroxide
O	Oxygen
OH	Hydroxide (Hydroxyl)
P	Product
PUFA	Poly-unsaturated fatty acids
S	Substrate
SCFA	Short-chain fatty acids
SFA	Saturated fatty acids
SH	Sulfhydryl group
TFA	Trans fatty acids
UFA	Unsaturated fatty acids
VFA	Volatile fatty acids
VLCFA	Very-long-chain fatty acids
α	Alfa conformation
β	Beta conformation

Research chapters:

A	Biomel
ADC	Average daily cost
ADG	Average daily gain
B	FP-Biomel
BW	Body weight
C	Kalfpap
cm	Centimeter
D	FP-Kalfpap
DMI	Dry matter intake
FCR	Feed conversion ratio

FP	Fermented protein
GC	Girth circumference
HC	High carbohydrate levels
kg	Kilogram
L	Liter
MJ	Megajoule
ml	Milliliter
MR	Milk replacer
MRS (agar)	De Man–Rogosa–Sharpe agar
n (lowercase)	Sample size
N (uppercase)	Population size
P1	Phase 1
P2	Phase 2
PW	Predicted weights
R^2	Coefficient of determination
SC	Stander carbohydrate levels
SM	Starter meal
SP	Standard protein
SW	Scale weight
TP	Total phase
WD	Weight difference

Chapter 1
General Introduction

1.1 Milk Replacer Industry

The milk replacer industry contributes to animal production and plays a significant role in the animal husbandry sector (Göncü *et al*., 2023). Milk replacers refer to products that substitute or supplement mother's milk in the suckling phase of mammalian animals (Soberon *et al*., 2012). As these milk replacers are commonly used to replace natural milk, it is formulated to mimic the nutrient value of natural milk. Commercial milk replacers must therefore provide adequate nutrients to ensure the health and growth of suckling neonatal animals (Palczynski *et al*., 2020).

There are various reasons and situations in which milk replacers are used and the expanding demand has led to the rapid expansion of the milk replacer industry (Kertz *et al*., 2017). The use of milk replacers ensures a consistent supply of nutrients to young animals under circumstances where the milk of the dam is unavailable as in the commercial dairy industry. Milk replacers are also used to mitigate the risk of disease transmission from the dam to the offspring (Nielsen *et al*., 2008; Van Niekerk *et al*., 2021). Milk replacers are therefore formulated to provide optimal growth, increase productivity and can provide a more flexible management option for farmers. According to Akins (2016), milk replacers can supplement mother's milk and partially replace it, providing farmers with a way to control the cost of rearing animals. As the composition of milk of different species vary significantly (Gantner, 2015), milk replacers must be formulated for a specific species, providing a research and development opportunity.

1.2 International milk replacer market trends

The global milk replacer market was valued at $2.5 billion in 2020 and is projected to reach $3.5 billion by 2025, growing at a compound annual growth rate of 7.1% (Markets and Markets, 2020). This suggests a rise in the number of neonatal calves, lambs, piglets and foals that are reared on milk replacers following on a worldwide rise to higher levels of global meat and dairy production (Ritchie *et a*l., 2023). There are also a wide range of situations necessitating the use of specialized milk replacers. Locally the increase in poaching has led to the development of specialized milk replacer for species such as rhinos (*Ceratotherium simum;* Cruywagen *et al.,* 2024). Another factor driving the growth of the industry is the increasing availability and adoption of advanced milk replacer products, which offer improved nutrition and health benefits for young animals (Wilms *et al*., 2022).

One of the biggest challenges for the milk replacer industry is the high cost of raw materials used for producing milk replacers, resulting in high production cost. This necessitates further research and

development to develop lower cost milk replacers and higher quality raw materials to improve the financial viability of milk replacers for farmers and producers (Schubert *et al.*, 2022).

The largest contributing countries to the global milk replacer industry are the United States, China, Germany and Brazil (KBV Research, 2022). Based on volume the United States (US) has consistently been the dominant producer and user of milk replacers (KBV Research, 2022). The advanced dairy farming infrastructure in the US, coupled with a focus on calf nutrition and health, and a strong focus on research and product innovation contributed to the success of the US milk replacer industry (Njuki, 2022). The availability of resources and a robust distribution network further contribute to the large market for milk replacers in the United States (Mendy, 2022).

China has recently witnessed a remarkable growth in its dairy industry also driving the milk replacer market and establishing itself as a key global player (Yin *et al.,* 2023). This is closely tied to the demand in the swine and dairy sectors (Hu *et al.*, 2023). China's burgeoning market can be attributed to the increasing demand for high-quality dairy products (Yin *et al.,* 2023), fuelled by new breeds, technology, improved animal health and nutrition, and supportive government policies (Bai *et al.*, 2018).

Germany holds a prominent position in the European milk replacer market and a significant portion of the milk replacer market is attributed to the dairy calf rearing industry. Schukat and Heise (2021) suggests that Germany's success is rooted in its willingness to embrace novel agricultural practices, stringent quality standards, and a strong commitment to animal welfare.

Brazil has also emerged as a significant player in the global milk replacer market, with a growing presence in the South American region. This leadership role in the global milk replacer market is propelled by their large beef cattle population and at 39.8 million dairy cattle, the second largest national dairy sector (Silva *et al*., 2019). Malafaia *et al.* (2021) highlights Brazil's expanding global market share, driven by the country's vast livestock industry, a focus on research and development, and increasing adoption of modern farming practices. Favourable climatic conditions and a rising demand for high-quality dairy products contribute to Brazil's robust milk replacer market (Picinin *et al.,* 2019).

1.3 Domestic (South Africa) milk replacer market trends

The South African milk replacer industry faces unique challenges including access to reliable information regarding the value of the milk replacer market in South Africa. The South African milk replacer market is comparatively small when compared to the global market. In monetary terms the market was estimated at between R30 and R35 billion per annum (Grobler, 2008), however due to the constantly changing economic environment and the lack of data the value may be significantly different. The South African industry have many small-scale farmers that often rely on barter trade and homemade products that does not necessarily directly add monetary value to the agriculture

sector. Although this might not be a significant portion of the South African milk replacer market the optimalisation of milk replacers and funding available from government can potentially assist small scale farmers in rearing calves.

One of the biggest challenges for this sector is the high production cost of milk replacers (Carulla *et al.,* 2023). Although this problem is not exclusive to the South African milk replacer industry, its impact is significant, affecting both small scale farmers (Syomiti *et al.,* 2014) and large commercial producers. The high cost of milk replacer can be directly attributed to the expensive, high quality raw materials needed to produce it (McCoard *et al*., 2021). This high-cost forces farmers to use less well-balanced alternatives such as whey sweepings and rejected baby milk which can negatively impact growth rates and overall productivity (Fischer *et al*., 2019; Creutzinger *et al*., 2021). Since a significant amount of milk replacers used in Southern Africa is developed and manufactured in first world countries, an unfavourable exchange rate further elevates cost challenges for imported milk replacers in the local market.

Despite these challenges, there is significant potential for growth and development within the milk replacer industry in South Africa. Especially in the dairy industry where it was found that the use of milk replacers in dairy calf rearing systems can lead to improved growth rates, and improved overall health and productivity (Uys *et al*., 2011; Huang *et al*., 2023). The use of milk replacers also has the benefit of reducing the risk of the transmission of diseases such as paratuberculosis and Johne's disease (Pieper *et al*., 2015; Vass-Bognár *et al*., 2022). This is possible as the use of milk replacers limits the chance of transmission between the mother and the neonatal calf.

To fully realize the potential of the milk replacer industry in South Africa, it is important to address the challenges facing the sector. In South Africa most of the hand reared calves originate from the dairy industry. Milk replacers are used to rear replacement heifers for dairies while terminal crossbred calves and Holstein bull calves are reared for meat purposes (Muller *et al.*, 2021). As explained, the cost of imported milk replacer limits their use for rearing bull calves and terminal crossbred calves, and locally produced milk replacers are needed to provide for this market. The types of milk replacers locally produced may differ between South Africa and other countries due to differences in the availability of raw materials, the cost thereof and production methods. To develop this sector, it is necessary to invest in research and development to improve the quality, affordability and accessibility of locally produced milk replacers. This may include investigating alternative sources of raw materials, developing more efficient production processes, and improving distribution channels to ensure that farmers have access to affordable and high-quality milk replacers.

1.4 Motivation

The fact that different milk replacers influence growth rate and the incidence of diarrhoea in calves is well documented (Amado *et al*., 2019; Johnson *et al.*, 2019). This effect differs depending on the

specific trial conditions and the parameters reported, while the use of different protein sources and different energy sources impacts on the cost of the milk replacer. The biological and economic viability of a specific feeding regime depends on growth rate, feed conversion, cost of the milk replacer and the incidence of growth insults caused by factors such as diarrhoea (Bach *et al.*, 2013; Hu *et al.*, 2019). In the current study the cost of the high carbohydrate milk replacer was significantly lower than conventional milk replacers and the cost of the fermented plant protein is also lower than commonly used alternative protein sources. Both alternatives therefore offer a considerable cost saving opportunity if animal performance could still be maintained.

1.5 Aims and Objectives

The size of the domestic and global milk replacer industry confirms the importance of this agricultural sector, highlighting the importance of research and improvement of milk replacers.

A study was conducted at the University of the Free State to determine:

- The effect of a high carbohydrate containing milk replacer on growth, general health and profitability parameters of Holstein dairy bull calves.
- The effect of a fermented plant protein containing milk replacer on growth, general health and profitability parameters of Holstein dairy bull calves. This was done as it was expected that during the fermentation process the amino acid composition of the plant protein would be changed as the plant protein would be degraded during fermentation and converted to primarily microbial protein with an amino acid profile closer to that of an animal protein.
- The impact of wean shock on Holstein bull caves when using high carbohydrate milk replacers.
- The effect of both a high carbohydrate and fermented plant protein containing milk replacer on growth, general health and profitability parameters of Holstein dairy bull calves.

The objectives of this study can be summarized as the evaluation of fermented plant protein as a novel protein source in calf milk replacers, using a calf rearing and growth model. The evaluation of milk replacers high in carbohydrates as alternatives to conventional milk replacers in calves as well as the efficacy of fermented plant protein in commercial and high carbohydrate milk replacers. Lastly a cost benefit analysis of the different milk replacers would determine the relative financial viability of each milk replacer.

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Chapter 2
Literature Review

2.1 Introduction

At birth, the calf's rumen is undeveloped and microbial fermentation does not play a role in the digestive process. Young calves are therefore referred to as pre-ruminants (Porter, 1969). During this phase the abomasum and duodenum are the main compartments where enzymatic digestion takes place and due to the reflex closure of the oesophageal groove, milk is directly shunted into the abomasum, bypassing the rumen (Ørskov, 1972). Even though milk does not directly affect rumen development and volatile fatty acids (VFA) produced during microbial fermentation, it plays an important role in the development of the rumen wall (Baldwin *et al.*, 2004).

The highest mortality rate in calves occurs during the weaning and pre-weaning phases, commonly caused by digestive diseases and disorders (Urie *et al.*, 2018). Passive immunity in neonatal calves is improved by the intake of colostrum from the dam (Weaver *et al.*, 2000). Passive immunity is commonly associated with the transfer of immunoglobulin G (IgG) from the dam's colostrum to the calf during the first few hours after birth (Tyler *et al.*, 1996). This occurs while the calf's digestive tract can non-selectively, through pinocytosis, absorb larger molecules, such as IgG, into the bloodstream (Stott *et al.*, 1979). The absorption of macro-molecules through the luminal wall gradually declines (Lecce *et al.*, 1964; Fischer *et al.*, 2018) and significant absorption ceases between 24 and 36 hours after birth due to changes in the luminal wall (Stott *et al.*, 1979). It is therefore recommended that the intake of colostrum should take place directly after birth and as frequently as possible during the first few hours to ensure sufficient passive immunity in calves (Fischer *et al.*, 2018). Proper nutrition also plays a role in sustaining the immunity of calves (Nonnecke *et al.*, 2003) and supports gut development, health, growth and productivity (Terré *et al.*, 2009). Intake of colostrum introduces bacteria into the small intestine of the young calf and the microbiomes that form in the gut positively influence gut health and overall performance (Malmuthuge *et al.*, 2015). As the number of bacteria are negatively correlated to absorption of immunoglobulins, a balance between these factors is important (James *et al.*, 1981). Signorini *et al.* (2012) have shown that the introduction of different *Lactobacillus* spp. during the weaning stage improves protection against infection and lowers the incidence of diarrhoea. Colostrum does not only contain immunoglobulins that are beneficial to the calf, but also sialylated oligosaccharides, growth factors, hormones, cytokines, enzymes, polyamines and nucleotides, antimicrobial components, and white blood cells (Hammon & Blum, 2002; Barile *et al.*, 2009; Langel *et al.*, 2015). Whey, commonly used in milk replacers, contain bovine sialylated oligosaccharides and glycoproteins such as lactoferrin and these substances have been shown to promote gut health in calves (Barile *et al.*, 2009; Zapata *et al.*, 2017).

When a calf is suckled by the dam, intake of transition milk after the colostrum phase occurs (Amaral-Phillips *et al.,* 2024). During this phase the milk composition gradually changes, unlike in a system where milk replacers are used, where the transition is mostly directly from colostrum to the milk replacer (Blum & Hammon, 2000). During the first weeks after birth, hand reared calves undergo multiple stressful situations due to radical changes in environment. These stress factors include the separation from the dam, vaccination programs, transport and housing changes and changes in feeding programs (De La Fuente *et al.*, 1999; Cho *et al.*, 2010; Urie *et al.*, 2018). This increases the probability and incidence of digestive disorders during this period (Urie *et al.*, 2018). Proper management, nutrition and colostrum consumption can contribute to lessening the impact of these detriments to calf health (Vasseur *et al.*, 2010; Costa *et al.*, 2016). Earlier it was common practice throughout the commercial industry to use milk replacers medicated with antibiotics. This practice has fallen out of favour due to legislation and the use of probiotics has become the norm (Wang *et al.,* 2023). Milk replacers containing probiotics has been reported to decrease the incidence of diarrhoea and improve growth rate in calves (Windeyer *et al.*, 2014; Wang *et al.,* 2023). Diarrhoea is a common indicator of these disorders and is a common problem in pre-weaned calves or calves having to adjust to new feeding protocols (Blanchard, 2012). Microbial additives such as lactic acid bacteria (mainly *Lactobacillus* spp.) and its fermented products can reduce diarrhoea in calves reducing the reliance on antibiotics (Signorini *et al.*, 2012). For effective use of microbial-based products in calves the interaction with colostrum, milk and starter meal should be considered as well as the condition of the calf and the digestion and absorption of the product (Malmuthuge *et al.*, 2015).

2.2 Mechanism and classification of enzymes

Closure of the oesophageal groove allows colostrum/milk/milk replacers to be shunted directly into the abomasum where it is digested through enzymatic digestion (Ørskov, 1972). In young calves the secretion patterns of enzymes have not stabilised, and dramatic changes take place during the first few days of life (Amin & Seifert, 2021). The pre-ruminant relies on enzymatic digestion where adult ruminants rely mainly on fermentation for the digestion of plant material as the rumen first must develop to support fermentation (Guo *et al.*, 2021). Thus, in pre-ruminants the function of enzymes as well as the secretion pattens of these enzymes greatly influence the ability to digest carbohydrates, proteins and fats. To be able to manipulate and optimise milk replacers for the best performance in young calves, the enzyme secretion patterns have to be considered (Hao *et al.*, 2021).

Enzymes govern many biological processes such as, transportation, respiration, excretion, reproduction, metabolism and digestion that occur in all tissues and fluids (Shilton, 2015; Dey *et al.*, 2019). Enzymes can be broadly defined as biological polymers that catalyses biochemical reactions without itself changing during the reaction (Robinson, 2015) and can be classified in terms of the type, structure, mechanism and various other factors affecting their activity (Martínez Cuesta *et al.*,

2015). Protein enzymes with catalytic capabilities are composed of linear chains of amino acids and have the typical three-dimensional structure of proteins (Lewis & Stone, 2023). The sequence of amino acids specifies the structure, which in turn identifies the catalytic activity of the enzyme (Robinson, 2015).

2.2.1 Enzyme mechanism

The basic mechanism of enzyme action is to catalyse a chemical reaction (Ribeiro *et al.,* 2023). This begins with the binding of the substrate to the active site of the enzyme where the enzyme provides the surface where the reaction occurs. The structure of the enzyme is characterized by hollow spaces where groups such as a sulfhydryl group (-SH) and carboxylic acid (-COOH) are found. In these areas the substrate combines with the enzyme and are referred to as the active sites (Robinson, 2015). This active site determines the specificity of the enzyme and it is not uncommon for this site to only accommodate a very specific substrate. The site is then referred to as site specific (Kuo *et al.*, 2016). For a successful catalytic reaction, the correct orientation and sufficient activation energy is required. During the transition state the substrate and the enzyme form an intermediate enzyme-substrate complex. While the enzyme remains unchanged, the substrate is changed through the binding and breaking of bonds, converting the substrate into the product. As the converted product is still bound to the enzyme it is referred to as the enzyme-product complex (Coey *et al.*, 2014; Liao *et al.*, 2011). The enzyme-product complex thereafter splits, releasing the product and enzyme. The free enzymes are then able to bind to the next substrate molecule and the catalytic cycle continues until all the substrate is converted (Robinson, 2015).

Compared to substrates, enzymes are typically large, varying in sizes, ranging from 62 amino acid residues to over 2500 residues in larger molecules and only a small section of the structure is involved in catalysis (Liu & Smith, 2021). The section involved in the catalysis is situated next to the binding sites (Gora *et al.*, 2013; Riziotis *et al.*, 2022). The active site on an enzyme is made up of the catalytic site and binding site. The catalytic residues of an enzyme are described as the amino acids that are directly involved in chemical catalysis and act as a general acid–base, electrophilic or nucleophilic catalyst, or they polarize and stabilize the transition state (Robinson, 2015). Figure 2.1 presents a visual presentation of the enzyme mechanism. The flow of free energy and kinetic energy in a reaction with and without the involvement of an enzyme is displayed, as well as a simplified structure to indicate the enzyme-substrate and enzyme-product, as it relates to the progress of the reaction.

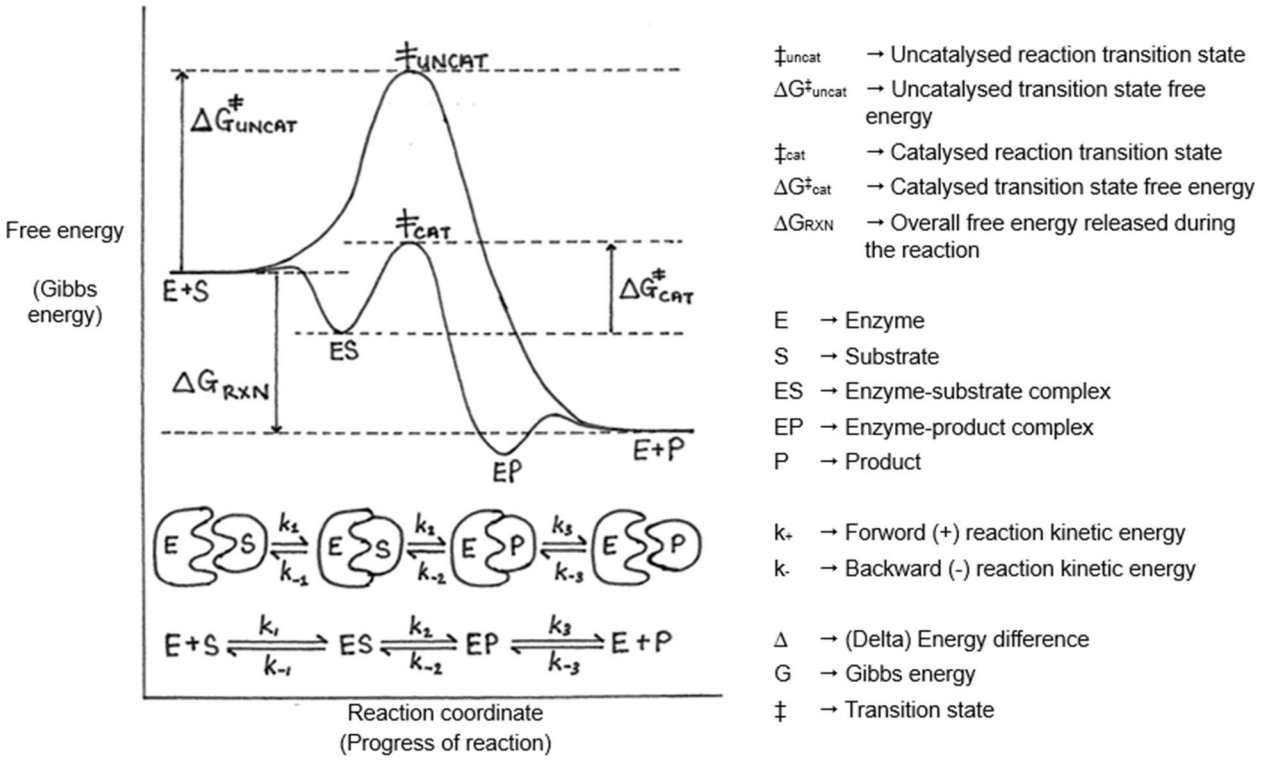

Figure 2.1 A graphical and simplified depiction of an enzyme mechanism adapted from Robinson (2015), Stivers & Nagarajan (2006) and Zhang *et al.* (2022).

2.2.2 Enzyme classification

Originally enzymes were assigned names based on the source or method of discovery, or by their discoverer (Martínez Cuesta *et al.*, 2015). This practice no longer applies, and a more comprehensive classification system is in use. According to the International Union of Biochemists, enzymes are divided into six functional classes and are classified based on the type of reaction that they catalyse (Martínez Cuesta *et al.*, 2015), this is listed in Table 2.1. The six kinds of enzymes are hydrolases, oxidoreductases, lyases, transferases, ligases and isomerases (De Souza Vandenberghe *et al.*, 2020). Although enzymes are classified according to the type of reaction they catalyse, enzymes can also be grouped based on other parameters such as the organ that secretes them. The types of classification used depends on the study or research it is applied to and should be clearly distinguished from the functional classes (Martínez Cuesta *et al.*, 2015; Karpińska & Czauderna, 2022).

The functioning of enzymes is often dependant on cofactors which are non-proteinoid substances that associate with enzymes (Bachosz *et al.,* 2023). An enzyme functioning without a cofactor is called an apoenzyme, while the combined enzyme and its cofactor constitute the holoenzyme (Robinson, 2015).

Other external factors that commonly influences enzyme activity is pressure, pH and temperature (Robinson, 2015). Enzymes perform their action most effectively at their optimum temperature and pH (Lin *et al.*, 2013). The temperature or pH at which a compound shows its maximum activity is

referred to as the optimum temperature or optimum pH, respectively (Robinson, 2015). As enzymes are protein compounds, their orientation is influenced by temperature and pH. This affects the shape of the molecule and its binding sites and influences their effectivity. This is especially important in the digestive tract environment where pH greatly influences the function and efficiency of digestive enzymes (Robinson, 2015; Wei *et al.*, 2022).

Table 2.1 The six functional classes of enzymes based on the reactions they catalyse with an example of each reaction (adapted from Kłos-Witkowska, 2015).

Functional class (Type)	Reaction catalysed. (Biochemical Property)
Oxidoreductase	Enzymes that catalyse oxidation reactions were the electrode(e^-) moves from molecule A to molecule B are classified as oxidoreductase. Example: $A^- + B \rightarrow A + B^-$
Transferase	Enzymes that help in the transportation of the functional group (B) from donor molecules (A) to acceptor molecules (C) are classified as transferase. Example: A-B + C → A + B-C
Hydrolases	Enzymes that catalyse hydrolysis reactions (a substitution reaction) by adding water to cleave the bond and hydrolyse it are classified as hydrolases (hydrolytic enzymes). Example: A-B H_2O → A-H + B-OH
Lyases	Enzymes responsible for catalysing addition and elimination reactions by breaking the bond between a carbon atom and another atom such as oxygen, sulphur, or another carbon atom thus the elimination of groups from the substrates by processes other than hydrolysis leaving (forming) double bonds. The reverse reaction is also catalysed (called a Michael reaction). Example: A-B → A=B + C-D | | C D
Isomerase	Enzymes that participate in the central metabolism of organisms and catalyses the rearrangement of the molecular structure of substrates. These enzymes catalyse the structural shifts present in a molecule, thus causing the change in the shape of the molecule, in other words they catalyse the formation of an isomer of a compound. Together with enzymes changing the redox state of substrates and transferring chemical groups between molecules, isomerases catalyse the interconversion of isomers i.e. molecules sharing the same atomic composition but different arrangements of chemical groups. Example: A-B → A-B | | | | C D D C
Ligase	Enzymes that catalyse the association of two molecules such as the ligation process where DNA ligase catalyse DNA fragments that form phosphodiester bonds. Example: A + B → A-B

2.2.3 Digestive enzymes

Digestive enzymes convert food into smaller molecules by breaking down polymeric macromolecules into smaller compounds to facilitate their absorption by the body (Sensoy, 2021).

These compounds play an important role in many metabolic functions. Enzymes break down carbohydrates to sugar, proteins to peptides and amino acids and fats to fatty acids (FA), cholesterol and glycerol (Goodman, 2010).

In this review enzymes are divided according to the type of nutrients they digest, although other authors group enzymes in accordance with the topic discussed, for example enzymes used in food processing industry (Ianiro *et al.*, 2016; Raveendran *et al.*, 2018).

When investigating digestion, digestive enzymes can be grouped by the feed fraction it breaks down, the specific function, the conformation and structure of the enzyme or the organ that secretes the enzymes. Therefore, there are multiple terms that can be used when investigating digestive enzymes, specifically enzymes responsible for the digestion of milk. The most important enzymes of interest within the digestive tract of the ruminant includes carbohydrase, amylase, protease, peptidase and lipase (Guo *et al.,* 2021).

2.2.3.1 Carbohydrase

Carbohydrases represent a group of enzymes, which is frequently used in the food industry (Contesini *et al.,* 2013). In the broadest sense, the term carbohydrase is considered to encompass diverse enzymes involved in hydrolysis and synthesis of carbohydrates, including amylase enzymes (Contesini *et al.*, 2013).

2.2.3.1.1 Amylase (Carbohydrase)

Amylase is a digestive enzyme predominantly secreted by the pancreas and salivary glands and is present in other tissues at minimal levels (Pieper-Bigelow *et al.*, 1990). The primary role of amylases is to break down the glycosidic bonds within starch molecules, transforming complex carbohydrates into simpler sugars (Fend, 2023). Amylase enzymes are categorized into three main classes namely alpha-, beta-, and gamma amylases with each of these classes targeting a distinct segment of the carbohydrate molecule (Zakowski & Bruns, 1985). Alpha amylase is present in humans, animals, plants, and microbes, whereas beta amylase is primarily found in microbes and plants. In contrast, gamma amylase can occur in both animals and plants (Azzopardi *et al.*, 2016).

2.2.3.2 Protease

Proteases are enzymes which catalyse the hydrolysis of peptide bonds between amino acids, present in proteins and polypeptides and includes enzymes such as trypsin, pepsin, and chymotrypsin (López-Otín & Bond, 2008; Mótyán *et al.,* 2013). Proteases can be classified based on their origin, catalytic activity and nature of the reactive group in the catalytic site (Raveendran *et al.*, 2018; Baharin *et al.*, 2022).

2.2.3.2.1 Exopeptidase (Protease)

The exopeptidases act on the ends of polypeptide chains and endopeptidases act randomly on the inner regions of polypeptide chains (Raveendran *et al.*, 2018).

2.2.3.2.2 Endopeptidase (Protease)

The endopeptidases are further classified into six groups, based on the catalytic residue present in the active site namely serine, aspartic, cysteine, metallo, glutamic acid and threonine protease (Raveendran *et al.*, 2018).

2.2.3.2.3 Peptidase (Protease)

Peptidases are enzymes capable of cleaving, and thereby often inactivating, small peptides (Baharin *et al.*, 2022). They are widely distributed on the surface of many different cell types, with the catalytic site exposed only on the external surface. Peptidases are involved in a variety of processes, including peptide-mediated inflammatory responses, stromal cell-dependent B lymphopoiesis, and T-cell activation (Yu *et al.,* 2022). In addition, some peptidases may have functions that are not based on their enzymatic activity (Van der Velden & Hulsmann, 1999). Many authors use protease and peptidase as synonyms and a few state that the terms are somewhat different as the proteases acts primarily by breaking down larger proteins into smaller peptides, while peptidases further degrade these peptides into individual amino acids or dipeptides (Mentlein, 2004). It should also be noted that some peptidases show both endopeptidase and exopeptidase activity (Barrett & McDonald, 1986).

2.2.3.3 Lipase

Lipases are enzymes which catalyse the hydrolysis of long-chain triglycerides (Pirahanchi & Sharma, 2024). They are secreted in the stomach and by the pancreas of humans and other animal species and digest fats and lipids (Raveendran *et al.*, 2018).

2.3 Digestive tract and enzymes

To understand the digestion of milk and milk replacers in ruminants it is important to grasp the layout, compartments and functions of the ruminant digestive tract as well the location where each enzyme functions. Although the focus in digestive studies is mostly on the rumen-reticulum and the abomasum, the rest of the digestive tract also plays a role in digestion and absorption of nutrients (Liu *et al*., 2021). The working of enzymes in the digestive tract can be either approached from the breakdown of specific feed components or from the flow through the digestive tract. The following

section will briefly review the intestines' primary functions and the different enzymes to simplify the explanation of location and processes in later sections.

Food is firstly ingested in the mouth and the ingesta is mixed with saliva (Sensoy, 2021). Ruminant saliva contains 99% water, and the solids consists primarily of sodium (Na) and potassium (K) salts that acts as buffering agents (Iorgulescu, 2009; Gokul, 2019). The saliva buffers the rumen and helps to keep the pH in a range of between 6.2 – 6.8 (Counotte *et al.*, 1979; Castillo-Lopez *et al.*, 2021). The specific pH differs depending on the diet and tends to be more acidic when ruminants consume high carbohydrate diets such as feedlot diets and more basic when more roughage is consumed (Ramos *et al.*, 2021). In monogastric animals the salivatory gland secretes lipase (salivatory lipase) which breaks down lipids (fat digestion) and amylase (salivatory amylase) which breaks down carbohydrates (Peyrot des Gachons & Breslin, 2016; Lai *et al.*, 2019). In ruminants salivatory amylase is however not present and carbohydrate digestion does not start in the oral cavity (McDougall, 1948; Vargas-Rodriguez *et al.*, 2014).

The rumen acts as a storage and fermentation vat and provides a chamber where archaea, viruses, fungi, protozoa and bacteria play a role in a microbial fermentation process (Matthews *et al.*, 2019). The temperature in the rumen ranges between 38-42°C (Rose-Dye *et al.*, 2011; Rutherford *et al.*, 2019) and different microorganisms in the rumen digest nutrients such as starch, sugars, proteins, oils and cellulose. The rumen wall is adapted for the absorption of digested nutrients through papillae which are tiny finger-like protrusions that cover the internal surface and significantly increases the area of the rumen wall (Gelberg, 2017). During microbial fermentation carbohydrates are degraded by microbial enzymes to simple sugars and volatile fatty acids (VFAs; Inman, 2011; Wang *et al.*, 2020). The most important VFAs are acetate (mainly used for fat synthesis), propionate (used for glucose synthesis) and butyrate (energy source of the rumen epithelium; Leng *et al.*, 1967; Bergman, 1990; Rémond *et al.*, 1995; Urrutia & Harvatine, 2017). During this fermentation process, gasses including carbon dioxide (CO_2) and methane (CH_4) are produced as byproducts (Chojnacka *et al.*, 2015; Morsy *et al.*, 2022). As there is no physical barrier between the rumen and reticulum, the ingesta can flow freely between the rumen and reticulum. These compartments are often grouped together and referred to as the reticulo-rumen (Wierzbicka *et al.*, 2021). The interior of the reticulum exhibits a honeycomb structure of strong muscle folds of different sizes that acts as a filter, allowing smaller digesta particles to move to the omasum and preventing food particles larger than 1 mm from leaving the reticulo-rumen (Kaske *et al.*, 1992; Gelberg, 2017). Although the reticulo-rumen does not play a significant role in enzymatic digestion of milk replacers in the pre-ruminant, the development of these organs is important to prevent weight loss during the weaning phase (Diao *et al.*, 2019; Miyazaki *et al.*, 2019; Schwarzkopf *et al.*, 2022).

The primary function of the omasum is water absorption (Holtenius & Björnhag, 1989). Digesta flows from the omasum and enters the abomasum through the oesophageal sphincter (Miyazaki *et al.*, 2019; Sensoy, 2021).

Enzymatic digestion in the ruminant starts primarily in the abomasum (Xu *et al.,* 2021) which will be discussed in detail in later sections of this review. The digesta flows from the abomasum through the pyloric sphincter into the small intestine (Sensoy, 2021). The monogastric stomach is equivalent to the abomasum in the ruminant and contributes significantly to enzymatic digestion of milk replacers in pre-ruminants (Gaowa *et al.,* 2021; Abdelsattar *et al.,* 2023). Enzymatic protein digestion starts in the abomasum when pepsinogen, a pro-enzyme, is secreted by the chief cells and converted to active pepsin by the gastric acid (Diao *et al.*, 2019; Kurz & Seifert, 2021).

The small intestine is divided into three sections, the duodenum, jejunum and ileum (Hickey *et al.,* 2023; Collins *et al.,* 2024). Digesta entering the small intestine are mixed with pancreatic- and brush border enzymes and bile, causing the pH to change from between 2.5 and 3.3 to between 7 and 8 (Capuano, 2017). At this pH the enzymes in the small intestine function optimally (Montoro-Huguet *et al.*, 2021). Active nutrient absorption occurs throughout the small intestine, including proteins that were not broken down in the rumen as well as microbial protein which are digested to amino acids in the abomasum and duodenum (He *et al.*, 2018). The intestinal wall of the small intestine is covered with villi which increases the surface area for improved nutrient absorption (Kiela & Ghishan, 2016). Once absorbed into the villi, nutrients enter the blood and lymphatic systems (Kohan *et al.*, 2011). In terms of enzymatic digestion, the small intestine (especially the duodenum) is the most important section of the digestive tract as the ducts transporting enzymes from the pancreas and bile from the bile bladder opens at the anterior of the duodenum (A-Kader & Ghishan, 2012). This enzymatic action is further enhanced by secretion of the brush border enzymes of the lumen wall (Holmes & Lobley, 1989; Hooton *et al.*, 2015). The pancreatic juices secreted into the duodenum contain pancreatic amylase, important for carbohydrate digestion in the pre-ruminant (Mellanby, 1925; Date *et al.*, 2015). The pancreas also secretes trypsinogen, chymotrypsinogen, pro-elastase and pro-carboxypetidase which play a role in protein digestion (Kobayashi, 1978; Szmola *et al.*, 2011; Tóth *et al.*, 2017; Vertiprakhov & Ovchinnikova, 2022) and lipase, responsible for fat digestion (Karpińska & Czauderna, 2022). The brush border enzymes responsible for carbohydrate digestion includes sucrase, maltase and isomaltase (Tannous *et al.*, 2023) while those involved in protein digestion includes enterokinase, enteropeptidase and peptidase enzymes such as amino peptidase and dipeptidase (Ozorio *et al.*, 2020).

The large intestine's main function is to act as a storage organ and the absorption of water and minerals. Secondary microbial fermentation, mostly of fibre, takes place in the large intestine and this, in the form of VFAs, provide between 10-15% of the total energy requirement of cattle (Liu *et al.*, 2023). No significant enzymatic digestion occurs here in monogastric, ruminant or pre-ruminant animals. The undigested feed components pass through the large intestine to the rectum and is excreted as faeces. In ruminants the cecum has a very limited function, differing from hind gut fermenters such as the horse where the cecum plays an important role in digestion (Sprekeler *et al.*, 2011; Chaucheyras-Durand *et al.*, 2022).

Figure 2.2 provides perspective of the flow of digesta through the digestive tract and where specific enzymes occur in the digestive tract. As depicted in Figure 2.2, the ruminant stomach consists of four compartments, the rumen, reticulum, omasum and abomasum. The closure of the oesophageal groove plays a critical role in suckling calves as it is responsible for the shunting of milk and milk replacers directly from the oesophagus to the abomasum, bypassing the rumen-reticulum (Kaba *et al.*, 2018). The oesophageal groove, also referred to as the reticular groove, is formed by muscular folds in the reticulum (Pokhrel & Jiang, 2024). Figure 2.2 compares the monogastric digestive tract with the digestive tract of a neonatal ruminant. As can be seen in the neonatal tract the rumen is undeveloped and the neonate ruminant is functionally a monogastric animal and depends on enzymatic digestion in the abomasum and duodenum (Diao *et al.*, 2019; Malmuthuge *et al.*, 2019). The rumen is undeveloped at birth and have to develop and be populated with micro-organisms before it becomes functional as a fermentation vat (Diao *et al.*, 2019; Su *et al.*, 2022).

Unlike monogastric animals, ruminants can utilize high forage-base diets due to the microbial fermentation that takes place in the rumen. During this process plant material is broken down by microorganisms and this provides nutrients and energy to the ruminants (Cammack *et al.*, 2018). As this is the primary way in which ruminants obtain their nutrients, studies often focus on the optimisation of nutrients for microbial fermentation as the simple rule of “look after the rumen microorganisms and they will look after the cow” is commonly applied. However, it has been determined that the optimisation of nutrition for young calves that relies mostly on enzymatic digestion in the abomasum directly influences adult ruminant performances (Diao *et al.*, 2019; Fischer *et al.*, 2019). Therefore, the optimisation of pre-ruminant diets is as important as the provision of optimal nutrients for adult ruminants.

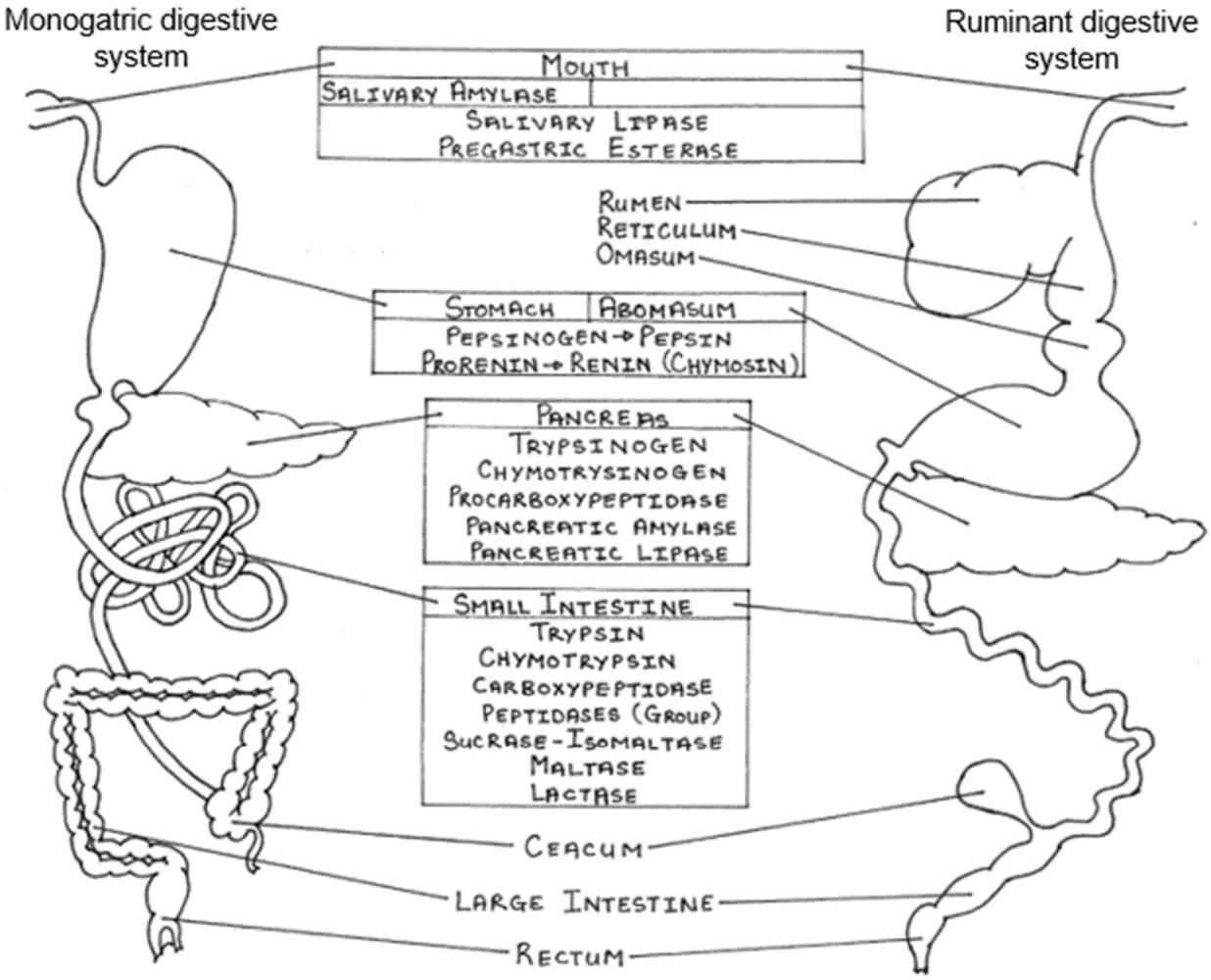

Figure 2.2 An animated and simplified depiction of digestive enzymes in the monogastric and neonatal ruminate digestive tract (combined and adapted from Amin & Seifert, 2021; Sensoy, 2021 and Tian *et al.,* 2023).

2.4 Digestion in the pre-ruminant

2.4.1 Carbohydrate digestion

According to Zhang *et al.* (2021a), carbohydrates are organic molecules that consist of carbon (C), hydrogen (H) and oxygen (O) atoms with a general chemical formula of $C_x(H_2O)_y$. Carbohydrates are commonly divided into four groups namely monosaccharides, disaccharides, oligosaccharides and polysaccharides (Navarro *et al.,* 2019). Monosaccharides, known as simple sugars, contain between three and nine carbon atoms and are classified as "*mono*-", referring to their single ring structure formation (NEWC, 2022). Carbohydrates in different conformations can be referred to as α-carbohydrates or β-carbohydrates. If the hydroxide (OH) group of the first carbon (C1) of the monosaccharide points in the opposite direction of the CH_2OH group on the last carbon in the monosaccharide ring it is referred to as the alfa (α) conformation, but if it points in the same direction the conformation is referred to as beta (β) (Zhang & Zhang, 1999). Glucose, fructose and galactose are examples of monosaccharide isomers with the same molecular formula of $C_6H_{12}O_6$ but with different structural conformations (Yang *et al*., 2016). The combination of two monosaccharides

forms a disaccharide and the “di-” refers to the two linked ring structures. Common disaccharides include sucrose, lactose and maltose (NEWC, 2022). Sucrose is composed of α-glucose and β-fructose linked by a α-1, β-2 glycosidic bond and this molecule is synthesised by plants, but not by animals (Navarro *et al.*, 2019). The bond between β-galactose and β-glucose is a β-1,4 glycoside bond (Leksmono *et al.,* 2018; Sadovnikova *et al.*, 2021; Dominici *et al.*, 2022). Lactose (Figure 2.3) is the most prominent carbohydrate in milk (Dominici *et al.*, 2022).

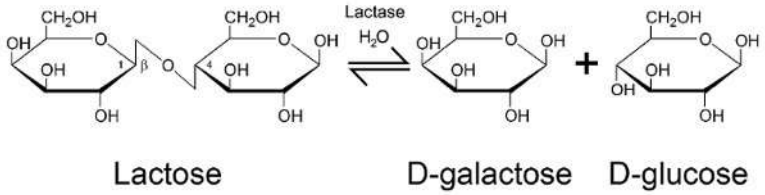

Figure 2.3 Molecular structure of lactose, D-galactose and D-glucose (Leksmono *et al.,* 2018).

Oligosaccharides are short chain monosaccharides containing less that twenty monosaccharides (Meyer *et al*., 2015).

Polysaccharides again are divided into homopolysaccharides, containing only a single type of monosaccharide and heteropolysaccharides, which contain two or more types of monosaccharides. Both groups can be branched or unbranched, with the places where the molecules branch referred to as the branch point (Khan *et al.*, 2022; Murphy *et al.*, 2023; Vetter, 2023).

Starch, dextran, glycogen and cellulose are all homopolysaccharides with glucose as building block (Tetlow & Bertoft, 2020). Starch, the carbohydrate produced and stored in plants, branch every 24 to 30 glucose residues and glycogen, the carbohydrate produced and stored in animals, branch every 8 to 14 glucose residues (Zmasek & Godzik, 2014; Pfister & Zeeman, 2016; Cho & Kang, 2020). Starch and glycogen contain α-1,4 glycosidic bonds and branch at α-1,6 glycosidic bonds (Brust *et al.,* 2020). Dextran is the structural component in bacteria and yeast and have α-1,6 glycosidic linkage that occasionally branch with α-1,2, α-1,3 and α-1,4 glycosidic linkages (Khalikova *et al.*, 2005; Froese *et al.*, 2015; Brust *et al.*, 2020). Cellulose is the structural component in plants and contains β-1,4 glycosidic bonds and hydrogen bonds and these bonds are hydrolysed by bacteria during fermentation, but cannot be hydrolysed by monogastric animals as these animals do not secrete cellulase (Behera *et al.*, 2017; Froidurot & Julliand, 2022).

Starch occurs in two forms of glucose polymers namely amylopectin and amylose (Van Zyl, 2017). Amylopectin is branched and the glucose molecules are linked by α-1,4 glycosidic bonds and α-1,6 glycosidic bonds. Amylose is unbranched and the glucose molecules are linked by α-1,4 glycosidic bonds (Bertoft, 2017; Van Zyl, 2017).

2.4.1.1 Carbohydrate digestive enzymes

As the saliva of ruminant animals does not contain amylase the enzymatic digestion of carbohydrates in pre-ruminants start in the small intestine (Vargas-Rodriguez *et al.*, 2014). This is unlike monogastric animals where it starts in the mouth due to the presence of α-amylase in the saliva (Boehlke *et al.*, 2015). In monogastric animals feed is therefore broken down both physically through mastication and enzymatically while in the ruminant only physical breakdown takes place.

Salivatory α-amylase in monogastric animals cleaves the α-1,4 glycosidic bonds through hydrolysis and this results in amylopectin and amylose (Boehlke *et al.*, 2015; Peyrot des Gachons & Breslin, 2016; Wang *et al.*, 2022a; Tian *et al.*, 2023). Due to the limited period at a neutral pH, salivatory α-amylase can only partially digest starch before the low pH in the stomach inhibits the working of this enzyme (Lovegrove *et al.*, 2017).

Although enzymatic digestion starts in the abomasum of the pre-ruminant no amylolytic enzymes are secreted in this region and therefore no significant carbohydrate digestion occurs here, and it only starts in the small intestine (Vargas-Rodriguez *et al.*, 2014; Hua *et al.*, 2022). The pancreas secretes enzymes via the pancreatic duct into the duodenum and the bile and pancreatic juice neutralises the pH to the extent that the secreted enzymes can function optimally (Tennant & Hornbuckle, 2014). The pancreas secretes α-amylase (pancreatic α-amylase) into the small intestine that hydrolyses α-1,4 glycosidic bonds. Starch is partially hydrolysed into oligosaccharides and shorter polysaccharides by pancreatic α-amylase and absorbed by the epithelial cells of the lumen wall (Date *et al.*, 2015; Kashtoh & Baek, 2023).

Figure 2.4 depicts starch digestion in the small intestine where starch is mostly digested and absorbed.

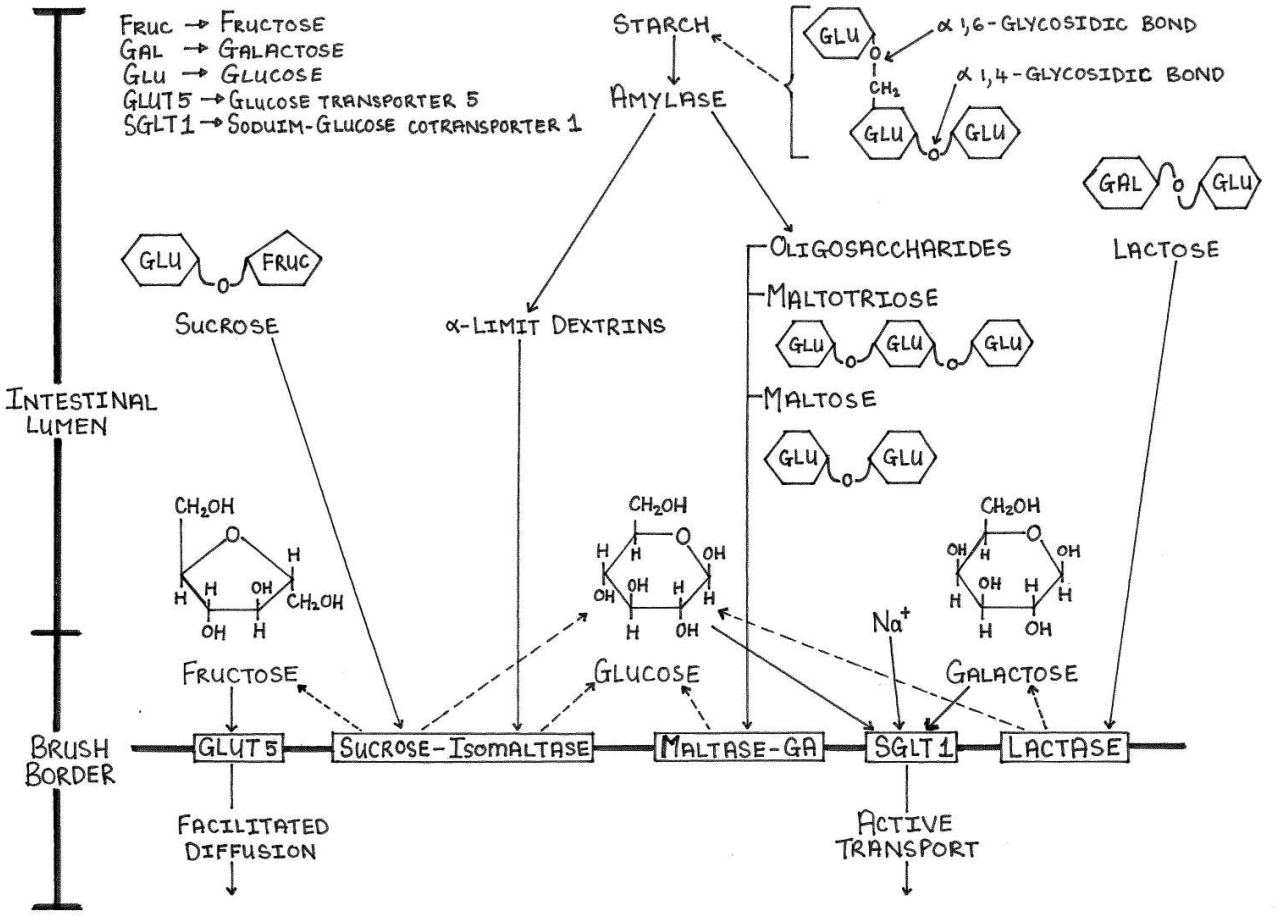

Figure 2.4 A simplified depiction of the major pathways in the small intestine for digestion and absorption of dietary carbohydrates, α-1,4 and α-1,6 glycosidic bonds of starch as well as the structure of simple sugars (adapted from Sibley (2004) and Navarro *et al.,* 2019).

Both salivary α-amylase and pancreatic α-amylase are extracellular enzymes capable of catalysing and hydrolysing α-1,4 glycosidic bonds found in starch, releasing glucose subunits (Freitas *et al*., 2017). In ruminants, pancreatic α-amylase digests starch to maltose and maltotriose (2- and 3- α-1,4 linked molecules, respectively) and α-dextrin (Guo *et al.,* 2021). As the sub-units of α-dextrin are linked with containing 1,6 glycosidic bonds these bonds cannot be hydrolysed by amylase (Aizawa *et al*., 2021).

2.4.1.1.1 α-Dextrinase

Isomaltase, also called α-dextrinase, acts as a debranching enzyme as it breaks α-1,6 bonds (Goodman, 2010). Isomaltase is the only enzyme capable of hydrolysing the α-1,6 glycosidic linkage in the α-limit dextrines (Livesey, 2014). A clear distinction should be made between α-dextrinase and thus isomaltase and α-limit dextrins. As α-dextrinase is an enzyme, whereas α-limit dextrin is formed when starch is digested with amylase and is comprised of glucose molecules linked with α-1,6 glycosidic bonds. Oligosaccharidase is also a product of starch digestion by amylase but only contain α-1,4 glycosidic bonds. The free glucose products of oligosaccharidase hydrolysis are transported into the enterocyte as described in the following sections. Isomaltase is part of a structure referred

to as sucrace-isomaltase in the small intestine (Senftleber *et al.,* 2023). This structure consists of two domains, the sucrase and isomaltase domains. This enzyme is anchored to the brush-border membrane in the small intestine. The isomaltase domain hydrolyses α-1,6 glycosidic linkages in isomaltose and oligosaccides derived from amylase-cleaved starch and glycogen (Senftleber *et al.*, 2023).

2.4.1.1.2 Maltase

Maltases are specialised α-glucosidases that catalyses the hydrolysis of the disaccharide maltose to the simple sugar glucose (Andriotis *et al.*, 2016). Maltase is a brush border enzyme in the small intestine that cleaves maltose by hydrolysing α-1,4 glycosidic bonds (Rose *et al.*, 2018). The natural form of maltase is maltase-glucoamylase (Maltase-GA) and removes single glucose residues from α-1,4 chains of oligosaccharides and form maltotriose and maltose (Peyrot des Gachons & Breslin, 2016).

2.4.1.1.3 Sucrase

Sucrase-isomaltase has two homologous functional subunits, sucrase and isomaltase. Both belong to the glycoside hydrolase family and differ in substrate specificity. Sucrase-isomaltase located on the brush border of the small intestine and the sucrase subunit cleaves the α-1,2 glycosidic bond in sucrose to produce glucose and fructose (Gericke *et al.*, 2017; Elferink *et al.*, 2020). According to Cohen (2016), sucrase-isomaltase is initially synthesized in the enterocyte as a single glycoprotein chain and, after insertion in the brush-border membrane, it is cleaved into sucrase and isomaltase units that reassociate non-covalently.

2.4.1.1.4 Lactase

Lactase is also referred to as β-galactosidase (Saqib *et al.*, 2017). Lactase belongs to the glycoside hydrolase family and catalyse the digestion of lactose (milk sugar) into glucose and galactose by cleaving the β-1,4 glycosidic bond between these molecules (Forsgård, 2019). This enzyme is therefore essential for the complete digestion of whole milk. Lactase is particularly abundant during infancy and is a brush border enzyme, produced by enterocytes cells that line the intestinal walls of the small intestine (Gerbault *et al.*, 2011; Forsgård, 2019).

2.4.1.2 High carbohydrate milk replacers

Lactose, commonly referred to as milk sugar, is the carbohydrate naturally found in milk and it must be hydrolysed before it can be absorbed (Dollar & Porter, 1957; Blaxter, 1988; Roy, 1990). Beta-galactosidase is responsible for lactose hydrolyses in the small intestine (Juers *et al.*, 2012). The

secretion of β-galactosidase increases shortly after birth and declines with age with a tenfold higher secretion in 8-week-old calves compared to adult ruminants (Coombe & Siddons, 1973; Toofanian *et al.*, 1973; Huërou-Luron, 2002). Pre-ruminants can readily utilize glucose, galactose and lactose, but has a limited ability to utilize maltose and starches and is unable to utilise sucrose (Dollar & Porter, 1957; Walker, 1959; Estévez *et al*., 2014).

In high carbohydrate milk replacers, cooked maize is used as the main source of carbohydrates. The building blocks of starch are glucose molecules and if starch is broken down to oligosaccharides and disaccharides, maltotriose and maltose results (Salema-Oom *et al.*, 2005; Damager *et al.*, 2010). Maltase and isomaltase activity therefore directly influence the rate at which starch is digested and the extent to which it can be utilized by neonatal and young calves. During the first four weeks of life, maltase and isomaltase activity increase, reaching adult levels (Le Huerou *et al*., 1992). In the adult ruminant, isomaltase activity is half that of maltase activity (Coombe & Siddons, 1973; Toofanian *et al.*, 1973; Le Huerou *et al.*, 1992).

The rate of starch digestion is determined by physicochemical characteristics such as granule size, degree of crystallinity, amylose: amylopectin ratio and the presence of other compounds (Parada & Aguilera, 2011; Van Zyl, 2017). Starch is also broken down in the large intestine by microbial activity (Serena *et al.*, 2008). Excessive intake of starch and any sugar, even lactose and glucose, by pre-ruminant calves, can cause diarrhoea. There is an upper limit to the amount of glucose that can be absorbed from the small intestine of the pre-ruminant (Velu *et al.*, 1960; Ballard & Oliver, 1965; Trotta *et al*., 2022). Due to the elevated levels of carbohydrates in high carbohydrate milk replacers, maltase and isomaltase activity levels are important for determining the optimal age at which the transition can be made from a standard milk replacer to a high carbohydrate milk replacer. Maltose activity gradually increase and in two-week-old calves, the enzyme activity reaches half the level of activity of adult cattle (Krehbiel *et al.*, 1996; Le Huerou *et al.*, 1992). At this age the enzyme activity is as such that milk replacers with a high starch content can be fully utilized by suckling calves.

2.4.2 Protein digestion

Proteins are organic compounds with amino acids as building blocks (Kitadai & Maruyama, 2018). These polypeptide chains are of varying length and if composed of more than fifty amino acids they are classified as proteins (Numata, 2020). Shorter chains composed of fewer amino acids are either polypeptides or peptides (Hou *et al.,* 2017). Proteins are important as a structural component in animal tissues as well as in biochemical, immunological, transportation and hormonal processes (Morris *et al*., 2022). The amino acids are linked by peptide bonds to form a chain (Jacob & Monod, 1961). Twenty different amino acids are commonly found in proteins and these amino acids contain an amino group (-NH2) and a carboxyl group (-COOH; Reeds, 2000). The chemical properties of the side chain are specific to each amino acid and is used to classify each of these amino acids. Therefore, amino acids can be classified according to the composition of the side chain as shown in

Table 2.2. The role of the amino acid in a protein is linked to the side chains and is used to identify the interaction between amino acids. This influences the way in which the protein folds, its stability and activity (Georgiou, 2018; Zhou & Pang, 2018; Andino *et al.*, 2023).

Amino acids can also be classified as essential and non-essential, as shown in Table 2.3. Essential amino acids refer to amino acids that cannot be synthesised *de novo* and therefore must be consumed (Church *et al.,* 2020). Implying that, non-essential amino acids can be *de novo* synthesized and is therefore not necessarily essential in the diet (Choi & Coloff, 2019). This classification system is more commonly used in the formulation of feeds.

Table 2.2 A summary of the classification of amino acids according to side chain properties (Jimenez-Morales *et al.,* 2012; Shaikh & Shah, 2015).

Amino acid classification group	Amino acid
Nonpolar, Aliphatic	Glycine (Gly, G) Alanine (Ala, A) Valine (Val, V) Leucine (Leu, L) Isoleucine (Ile, I) Proline (Pro, P) Methionine (Met, M)
Aromatic	Phenylalanine (Phe, F) Tyrosine (Tyr, Y) Tryptophan (Trp, W)
Polar, Uncharged	Serine (Ser, S) Threonine (Thr, T) Cysteine (Cys, C) Asparagine (Asn, N) Glutamine (Gln, Q)
Positively charged (Basic)	Lysine (Lys, K) Arginine (Arg, R) Histidine (His, H)
Negatively charged (Acidic)	Aspartic acid (Asp, D) Glutamic acid (Glu, E)

The quality of protein is of great importance in the formulation of milk replacers and the quality of a protein is mainly defined as the ability of a specific protein to provide the necessary amino acids in the required ratio for optimal growth and development of the neonatal calf (Katz *et al.,* 2019; Schubert *et al.,* 2022). Milk proteins have a high apparent digestibility for the pre-ruminant and the absorbed amino acids are efficiently utilized for protein synthesis (Huang *et al.*, 2015).

Table 2.3 Amino acids classified according to essential and non-essential (Buford *et al.,* 2008).

Essential amino acids	Non-essential amino acids
	Alanine
Histidine	Arginine
Isoleucine	Asparagine
Leucine	Aspartic acid
Lysine	Cysteine
Methionine	Glutamic acid
Phenylalanine	Glutamine
Threonine	Glycine
Tryptophan	Proline
Valine	Serine*
	Tyrosine**

*Serine is generally classified as a nutritionally non-essential (dispensable) amino acid, but metabolically, serine is indispensable and plays an essential role in several cellular processes (Kalhan & Hanson, 2012). This led to some tables not including Serine at all since the classification non-essential amino acid can be disputed.
**Classical animal nutrition textbooks do not consider cysteine or tyrosine as essential amino acids, as they can be synthesized in the liver from methionine and phenylalanine respectively. However, the inability of all animals to form the carbon skeletons for methionine and phenylalanine means that there is no de novo synthesis of cysteine or tyrosine (Wu, 2014) This led to the same situation as with serine where some textbook tables completely exclude tyrosine.

2.4.2.1 Protein digestive enzymes

The movement of milk through the abomasum is slowed down by clotting (Cruywagen *et al.*, 1990). Chymosin or renin is a proteolytic enzyme related to pepsin and is secreted by the chief cells in the abomasum where it is primarily responsible for the coagulation of milk (Andrén, 2011; Bezie & Regasa, 2019). Pepsin and hydrochloric acid (HCl) contribute to a degree during this process (Miyazaki *et al.*, 2019). During the clotting process, the chief cells in the gastric mucosa of young ruminants secretes pro-renin, an inactive precursor to renin (Guilloteau *et al.*, 2009). Pro-renin is activated to renin by acid or enzymatic cleaving in the presence of calcium ions (Guang *et al.*, 2012). Renin binds to casein and milk fat, forming an abomasal clot (Yvon *et al.*, 1984). Clotting increases the time that digesta remains in the abomasum and results in a more controlled flow of chyme through the pyloric valve, improving digestion and absorption in the small intestine (Petit *et al.*, 1987; Longenbach & Heinrichs, 1998; Andrén, 2011). If clotting does not occur, it can lead to the too rapid emptying of the abomasum, increasing the incidence of diarrhoea in calves (Petit *et al.,* 1987; Cruywagen *et al.*, 1990). The abomasal clot is degraded by proteolytic enzymes and lactose and whey are expelled from the abomasum by the contraction of the clot and the normal contraction of the gut wall (Petit *et al.*, 1987).

Protein digestion involves the denaturing of proteins to expose peptide bonds allowing hydrolyzation of the molecule, and the release of free amino acids. Protein-digesting enzymes in the abomasum can be either endopeptidase or exopeptidase. Exopeptidases catalyse the cleavage at the N-terminal or C-terminal removing a single amino acid, while endopeptidases cleave peptide bonds within the sequence (Van der Velden & Hulsmann, 1999; López-Otín & Bond, 2008).

Pepsinogen is secreted by peptic cells in the oxyntic glands in the abomasum and by mucosal cells in the gastric antrum and the duodenum (Norris & Hersey, 1985; Rowbotham *et al.*, 2006). In the presence of hydrochloric acid (gastric acid) this proenzyme is converted into active pepsin, which also catalyses the further conversion of pepsinogen to pepsin (Richter *et al.*, 1998). The main stimulus for pepsinogen release is the increased vagal activity observed during the cephalic and gastric phases of acid secretion. Gastric acid also initiates a local cholinergic reflex that triggers pepsinogen secretion from peptic cells. When acidic gastric chyme flows into the duodenum it stimulates the release of secretin. Secretin and gastrin cause further pepsinogen secretion (Hirschowitz, 1984).

Protease enzymes, secreted in inactive form, is activated by a cascade in the duodenum. Enterokinase, a protease of the intestinal brush border of the duodenum cleaves pancreatic trypsinogen to yield active trypsin (Kitamoto *et al.,* 1994). Trypsin cleaves chymotrypsinogen to yield active chymotrypsin and these enzymes are responsible for the breakdown of proteins to polypeptides (Antalis *et al.*, 2007). Trypsin also cleaves procarboxypeptidase to yield active carboxypeptidase (Villegas *et al.*, 1995; Antalis *et al.*, 2007). Carboxypeptidase and dipeptidases cleaves polypeptides to single free amino acids that are absorbed through the membranes of the villi of the small intestine into the bloodstream (Antalis *et al.*, 2007).

2.4.2.2 Protein sources

Protein requirement of cattle depends on age, physiological status (e.g., pregnancy, lactation) and pathological status, as well as the quality of the protein supplied (NRC, 2021). It is further determined by the extent to which different feed ingredients supply essential nutrients needed for efficient, economical, and sustainable production (Beever, 1996). Dietary protein must supply adequate amounts of the required amino acids in the correct ratio (NRC, 2021). According to Ertl *et al.* (2016), the amino acid profile of the protein in the diet, the essential amino acids as well as the concentration of the limiting amino acids, the digestibility and physiological utilization of amino acids determine the value of a protein source. The ultimate quality of a protein can be defined as the ability of the ingested protein to meet the requirements for all the dietary indispensable amino acids in an animal and this can be measured by the Digestible Indispensable Amino Acid Score (DIAAS; Wolfe *et al.*, 2016). Bioavailability of amino acids can be defined as amino acids in a form suitable for digestion, absorption, and utilization and can only be estimated using animal growth bioassays with live animals (Jahan-Mihan *et al.*, 2011). The origin of protein, such as animal versus plant protein and feed processing methods, can affect protein digestibility, influencing bioavailability. Compared to plant protein, animal protein generally contains a better balance between essential amino acids and non-essential amino acids (Ertl *et al.*, 2016). Additionally, plant protein, such as soybean meal, often contain antinutritional factors (e.g., trypsin inhibitor) that can affect protein digestibility and availability of amino acids (Jahan-Mihan *et al.*, 2011). Feed processing methods such as heating, roasting, and

extrusion have however been shown to disrupt antinutritional factors like trypsin inhibitor in soybean meal (Akande & Fabiyi, 2010). According to El-shemy *et al.* (2000), overheating however render amino acids unavailable due to a complex reaction with sugars (Maillard reaction).

2.4.3 Fat digestion

The major energy source in milk in the pre-ruminant diet is fats or lipids (Guetouache *et al.*, 2014). The most common type of fat is triglycerides, referring to the glycerol backbone and the three fatty acids binding to it (Mills *et al.*, 2017). Fatty acids have a carbon back bone, and the length of the fatty acids is used to classify the different fatty acids. Short-chain fatty acids (SCFA) consist of up to six carbon atoms (Schönfeld & Wojtczak, 2016; Morales-Olvera *et al.*, 2021), medium-chain fatty acids (MCFA) of seven to twelve carbon atoms (Schönfeld & Wojtczak, 2016), long-chain fatty acids (LCFA) of thirteen to twenty-one carbon atoms, and very-long-chain fatty acids (VLCFA) of twenty-two and more carbon atoms (Batsale *et al*., 2021; Zheng *et al.*, 2017). Fatty acids are also classified according to the number of double bonds in the carbon chain (Satari *et al.*, 2018). Saturated fatty acids (SFA) have no double bonds in their carbon chain, where mono-unsaturated fatty acids (MUFA) have a single double bond (DiNicolantonio & O'Keefe, 2022). Poly-unsaturated fatty acids (PUFA) refer to lipids containing more than one double bond in their carbon chain (Sokoła-Wysoczańska *et al*., 2018). Poly-unsaturated fatty acids are further divided according to the first double bond from the methyl-end on the opposite side of the glycerol backbone into three groups. Omega-3 (n-3) fatty acids contain a double bond on the third carbon (between the third and the fourth carbon), omega-6 (n-6) fatty acids contain a double bond on the sixth carbon (Sokoła-Wysoczańska *et al*., 2018) while omega-9 (n-9) fatty acids contain a double bond on the ninth carbon (Gonçalves-de-Albuquerque *et al.*, 2016). Unsaturated fatty acids (UFA) are further classified according to their conformation since the double bonds can change the linear form of the carbon chain. If the hydrogen on the carbons on either side of the double bond is on the same side, the conformation is classified as "cis-", resulting in a bend in the chain as the hydrogen molecules repel each other. If hydrogens are on opposite side the conformation is classified as "trans-", resulting in a linear structure of the fatty acid (Marchand, 2010). Cis fatty acids (CFA) are predominantly natural in origin, while trans fatty acids (TFA) are predominantly produced by industrial processes (Orsavova *et al.*, 2015; Hirata *et al*., 2023).

When considering fat in the diet, the conformation of fatty acids must be considered since it plays a role in the characteristics of the fat. A high PUFA concentration in milk replacers lead to poor growth and digestibility and may also increase the incidence of diarrhoea in calves compared to medium-chain to long-chain SFAs and long-chain MUFAs (Jenkins *et al.*, 1985). Supplementation of n-3 FA in milk replacers can also lead to a decrease in starter intake and weight gain (McDonnell *et al.,* 2019) when compared with the bovine milk, which contains medium-chain to long-chain SFA and long-chain MUFA. Butyric acid (C4:0), a SCFA (Glass *et al.*, 1967), have many positive effects and

was reported to promote the development of the digestive tract (Górka *et al.*, 2018). It is also highly digestible by pre-weaned calves and improves pancreatic secretion and digestibility (Guilloteau *et al.*, 2010).

On a dry matter (DM) basis, the butterfat content of cow's milk contributes between 25 and 30% of the dry matter and is highly digestible (Mirzadeh *et al.*, 2010; Mourad *et al.*, 2014). If this nutrient is replaced with non-natural fats and oils in milk replacers, it can lead to a reduction in digestibility (Pluschke *et al.*, 2016). Interesterification, which refers to the random rearrangement of the constituent fatty acids on the glycerol molecule, improves the digestibility of fats in milk replacers in most instances, but this do not apply for butterfat (Hamilton & Raven, 1972). This suggests that high digestibility is not dependent on any specific triglyceride structure. It is important to note that high-quality protein is necessary for high fat digestibility in milk replacers. Therefore, except for butterfat, if the quantity and quality of protein is reduced there will be a reduction in digestibility of most fats (Gibney & Walker, 1977).

According to Gibney & Walker (1977) and Liu *et al.* (2014) the factors responsible for reduction in digestibility of vegetable oils and animal fats in pre-ruminants includes:

- The chain length of the constituent fatty acid
- The degree of saturation of the fatty acids
- The positioning of individual fatty acids on the glycerol molecule
- The proportion of saturated triglycerides in the fat
- The absence of high-quality protein

2.4.3.1 Fat digestive enzymes

Pre-ruminant and monogastric fat digestion are similar and starts with a process of hydrolysis (Ramsey *et al.*, 1956; Grosskopf, 1965; Diao *et al.*, 2019), which refers to the cleavage of chemical bonds by the addition of water. Fat digestion and carbohydrate digestion occur concurrently. Fat digestion starts in the oral cavity (Choi & Snider, 2019) with the secretion of salivary lipase and pre-gastric esterase (Hamosh, 2019). At one week of age, it is estimated that pre-gastric esterase activity is sufficient to hydrolyse between 65-70% of the lipid offered in a milk diet (Gooden & Lascelles, 1973). The net result of the action of the pre-gastric esterase is an accumulation of free fatty acids in the abomasum (Edwards-Webb & Thompson, 1977). The extent of lipolysis is however limited by the inability to release long-chain fatty acids before the chyme becomes too acidic in the abomasum (Lai *et al.*, 1997).

When entering the duodenum, dietary lipids (mostly triglycerides) are emulsified by bile salt released from the gall bladder (Maldonado-Valderrama *et al.*, 2011; Pitt & Gadacz, 2013). Due to their hydroxyl (OH) and carboxyl (COOH) groups, bile salt functions as a detergent and this causes large lipid molecules to form smaller lipid droplets surrounded by a layer of bile (Di Gregorio *et al.*, 2021;

Durník *et al.*, 2022). The emulsified lipids are digested by pancreatic lipase and converted into fatty acids, monoglycerides and glycerol and assembled into micelles (Brobst, 1980; Iqbal & Hussain, 2009). Micelles are formed by the short-term combination of bile salt, fatty acids, monoglycerides, and other fat-soluble substances such as vitamins and cholesterol (Reshetnyak, 2013). The micelles are water soluble (Lukyanov & Torchilin, 2004), and this enables their transportation to the small intestine surface where they are absorbed. At the site of absorption, the micelle breaks down and the bile salt returns to the intestine to repeat the emulsification processes (Bodewes *et al.*, 2015). The components of the micelles are absorbed into the small intestine by passive diffusion (Iqbal & Hussain, 2009). Saturated fatty acids forms micelles less efficiently than unsaturated fatty acids (Cherian, 2019). For this reason, a blend of saturated and unsaturated fatty acids should be used in milk replacers (Cherian, 2019). Once inside the intestinal cell (enterocyte), the monoglycerides and fatty acids are re-esterified, and in combination with free and esterified cholesterol, lipoproteins and phospholipids chylomicrons are formed. The chylomicrons are then secreted into the lymphatic system (Cherian, 2019).

2.4.3.2 Fat inclusion levels in milk replacers

Milk replacers is formulated to mimic natural milk, with the aim to optimise animal growth performances at the lowest possible cost. As dietary fat significantly affects the functioning of the rumen, many studies reported on the inclusion of fat in starter meals rather than in milk replacers (Berends *et al*., 2018; Ghorbani *et al*., 2020). Consequently, specific fat limitations are imposed to ensure optimal rumen development without compromising overall calf health (Amado *et al.,* 2022). However, as suckling calves are less sensitive to the level of fat inclusion and optimal inclusion level in milk replacers is not well established as it is influenced by factors such as breed, environment and enzymatic secretion pattern (Echeverry-Munera *et al.,* 2021; Wilms *et. al.,* 2024). Researchers have also investigated the effects of varying fat levels on calf growth and nutrient uptake as fat provides a key source of energy and plays an essential role in the absorption of fat-soluble vitamins (Youness *et al*., 2022).

Sharma *et al*. (2020a) reported reduced dry matter intake when feeding milk replacers to dairy calves with fat content of 14% versus 10%. The different levels of fat and protein in the ration however did not affect average daily gain in calves (Sharma *et al*., 2020a). Pancreatic lipase enzyme secretion of pre-weaned calves is influenced by both age and dietary fat content (Harmon, 1992). Górka *et al*. (2014) have shown that the activity of digestive enzymes such as amylase, lipase and protease increase with age in young calves.

Overall, most studies suggest that the activity of digestive enzymes in young calves are influenced by both age and dietary fat content. While there is no clear consensus on the optimal level of fat in milk replacers for calves, literature suggest (Hill *et al.*, 2011) that a range of fat concentrations may be suitable, and that the specific concentration may depend on other factors such as the nutritional

composition of the milk replacer, the age and weight of the calf, and its health status. These findings also suggest that increasing the dietary fat level can improve the activity of pancreatic lipase, but the degree of this effect may depend on the age of the calf (Hill *et al.*, 2009).

Fermented plant protein has a fat content of 20% (Cordy, 2024). This implies that the source and composition of fat will be different between the commercial milk replacer and the milk replacers formulated with fermented plant protein.

2.5 Raw material processing and milk replacer comparisons

Processing methods of raw materials can vary widely depending on the industry and the aim for using the method. Multiple methods are used for food or raw material processing and for different applications. These processing techniques can further be broadly divided according to the type of application (Huss *et al.*, 2018; Michel *et al.*, 2024). In some cases, food treatment processes and raw material processing techniques can overlap whilst being exclusive in other applications. Amit *et al.* (2017) classified food processing according to factors affecting food spoilage, while Avilés-Gaxiola *et al.* (2018), classified processing methods of a raw material according to inactivation methods of trypsin inhibitor. Although studies like these are applicable in completely different scenarios it shows that the most comprehensive way to divide the processes are according to physical processing, chemical processing and biological (microbial in some cases) processing. Animal feed processing is commonly divided into wet and dry processing and further subdivided into hot and cold processing. Wet processing methods include grinding, dry rolling, flaking, pressure cooking, exploding, pelleting, reconstitution, extrusion, fermentation and gelatinization (Huss *et al.*, 2018). Dry processing methods include grinding, dry rolling, popping, micronizing, extruding and roasting, decorticating/dehulling and crumbling (Dattatray & Pramond, 2023).

The reason for feed processing may differ, but in general feed and raw material processing techniques and methods are used to increase the nutrient composition and/or utilization and/or the economic value thereof (Weaver *et al.*, 2014; Singh *et al.*, 2023). This can include processes that simplify storage, utilize raw materials that would otherwise be unavailable or change feedstuff composition (Van der Poel *et al.*, 2020). Figure 2.5 provides an overview of the categories of processing methods for raw materials that is commonly used when producing raw materials used in producing milk replacers.

Food processing is defined by Albuquerque *et al.* (2022) as the activities involved during the transformation of raw materials from different origins to achieve a final product suitable for consumption. Amit *et al.* (2017) defines food processing as merely a part of food preservation and states that food preservation involves different food processing steps to maintain food quality at a desired level so that the maximum benefits and nutrition values can be achieved. Van der Poel *et al.* (2020), focused more on animal feeds and define feed processing as techniques in which physical,

chemical, biochemical, biological and physiochemical methods are applied to increase the nutrient utilization of feeds and fodders. The aim is therefore to optimize nutritional value of feeds (Sarnklong *et al*., 2010).

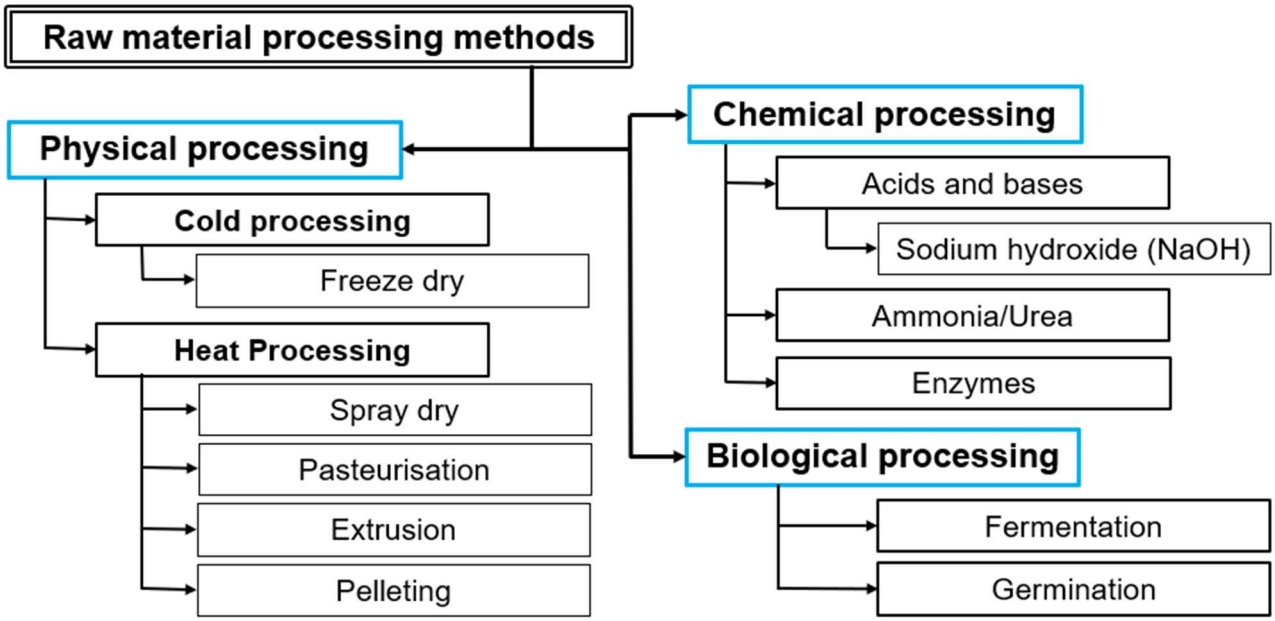

Figure 2.5 A brief overview of the different categories of processing methods for raw materials adapted from Van Zyl (2017) and Avilés-Gaxiola *et al.* (2018).

2.5.1 Physical processing

Physical processing can be divided into cold processing and heat processing although not all processes fit into these categories (Van Zyl, 2017). Physical processing in the food industry is commonly divided into thermal processing, non-thermal physical processing and physical detection (Ma, 2018). Freeze drying is categorised under cold processing and is of specific importance as it is often confused with spray drying which is a heat process and is commonly used when producing raw materials for milk replacers (Schuck, 2002; Baldelli *et al.*, 2022).

2.5.1.1 Freeze drying

Freeze-drying, also known as lyophilization, is often used to produce high-quality dehydrated fruits and vegetables (Nath *et al.,* 2023). During freeze-drying the product is frozen and the water is in solid-state (Nowak & Jakubczyk, 2020). The low temperature and moisture sublimation process preserve the fundamental structure and shape of the products, resulting in a final product which have a low bulk density, high porosity, and favourable rehydration characteristics (Bhatta *et al*., 2020). Sublimation of the liquid is achieved by subjecting the frozen substrate to a vacuum and this causes the solvents to sublimate (Qian & Zhang, 2011). The process can be broken down into three stages namely freezing, sublimation (also known as primary drying) and desorption (also known as secondary drying). During the sublimation stage most of the solvent is removed and during the

desorption stage the temperature increases to break the ionic bonds, and the remaining solvent is almost completely removed (Assegehegn *et al.*, 2019).

2.5.1.2 Spray drying

Spray drying is a heat processing method and is grouped in this discussion with other physical processing methods such as pasteurisation, extrusion and pelleting (Schuck, 2002). Heat processing is widely accepted as the most effective way of improving the overall nutritional value of legume seeds because it improves protein digestibility principally through the inactivation of thermolabile anti-nutritional factors (Avilés-Gaxiola *et al.*, 2018).

Spray drying is commonly used to produce raw materials used in the production of milk replacers (Schuck, 2002; O'Neill *et al.*, 2019). It is one of the most efficient ways to extend the shelf life of liquid extracts and improve the organoleptic features of products by converting extracts into a stable dry powder (Nath *et al.*, 2023). With spray drying, liquid feeds (extracts) are atomized into the drying chamber and the resulting droplets pass through a hot-air (or sometimes nitrogen) stream to evaporate the liquid fraction (Filková & Mujumdar, 2020). The evaporation of water takes place at a faster rate when the droplets themselves are smaller since this increases the surface-to-mass ratio (Santos *et al.*, 2017). Because of the speed and intensity with which this procedure is carried out, there is very little heat damage caused to sensitive material (Nath *et al.*, 2023).

2.5.1.3 Pasteurisation

Pasteurisation is a low order heat treatment and is often performed at a temperature below the boiling point of water (Iordache *et al.*, 2017). It can be divided into slow pasteurisation and rapid pasteurisation (Deak, 2014; Booker *et al.*, 2023). With slow pasteurization, the pasteurization time is linked to the temperature use. For a treatment temperature of between 63ºC and 65ºC, the period often exceeds 30 minutes and for a treatment temperature of 75°C a treatment period of between 8 to 10 minutes is used (Deak, 2014; EFSA *et al.*, 2021). With rapid, high or flash pasteurization the temperature range is between 85ºC to 90°C and the treatment period is a few seconds (Deak, 2014; Sfakianakis & Tzia, 2014).

The general objective of pasteurization is to extend product shelf-life by inactivating all non-spore-forming pathogenic bacteria and most vegetative spoilage microorganisms, as well as inhibiting or stopping microbial enzyme activity (Nguyen, 2023). To be effective, pasteurization is frequently combined with another means of preservation such as concentration, acidification and chemical inhibition (Deak, 2014). Although pasteurization inactivates pathogens, it results in the loss of immunological functions and bactericidal action in human milk (Patil *et al.*, 2019) and should therefore not be used as a processing method when the aim is the transference of antibodies to neonatal animals.

2.5.1.4 Extrusion

Extrusion is a continuous short-time cooking process and is therefore referred to as extrusion-cooking (Téllez-Morales & Rodríguez-Miranda, 2023). During the extrusion process the product is exposed to high temperatures ranging between 125°C - 170°C for a relatively short time of between 15-30 seconds (Rowe *et al.*, 1999). Extrusion-cooking can be described as a process whereby moistened starchy and/or proteinaceous materials, usually starchy grains, meals, and flours, are cooked and worked into a viscous, plastic-like dough (De Cruz *et al*., 2015). Rowe *et al.* (1999) describes the processes as food materials fed into a barrel containing a single or twin rotating screw(s). The material is forced to pass through a die plate that shapes extrudates into given configurations. The barrel is usually heated to gelatinize starches and enhance protein denaturation and reactions such as hydrolysis of peptide bonds at aspartic acid residues and interchange or destruction of disulfide bonds are involved. The screw configuration which imparts mechanical shear and die plate along with the temperatures applied in the different barrel sections affect the internal pressure and the degree of cooking (Luy *et al.*, 2022). One of the main advantages of this process is that the short treatment time maintains the nutritional value of extrudates (Boakye *et al*., 2023).

In modern poultry farming systems, the focus is on reducing the feed cost by enhancing feed efficiency by adopting modern processing techniques (George & George, 2023). The extrusion process is one of the techniques used to not only enhance the nutritional value of the ingredients and feed, but also the efficiency of utilization of the feed (Le Boucher, 2024). In modern feed milling operations, extrusion is considered a standard process used to enhance the value of a feed (Rahman *et al.*, 2015). The degree of enhancement due to an extrusion process depends upon factors such as structure and chemical composition of the ingredients, processing conditions and machinery used (Rojas *et al*., 2016). Variation in extruding temperature, moisture, screw speed, pressure, time, chemical composition and structure can influence the nutritional value, digestibility of feed or feed ingredients. This will affect the performance of the animal and to ensure optimal results from extrusion processing all conditioned should be maintained at optimum levels (Rahman *et al.*, 2015).

2.5.1.5 Pelleting

Pelleting of animal feeds to denature protein, increase starch gelatinization, decrease anti-nutrient factor content, and improve the nutritional value of feed ingredients has been extensively used for decades (Wang *et al.*, 2022b). It is not used to produce milk replacers, but is commonly used as a final step when manufacturing animal feeds and starter pellets for calves (Birger & Zimonja, 2011). Pelleting is the most prevalent heat treatment in the production of poultry feeds. However, due to the heat, moisture and mechanical pressure applied during pelleting, some chemical and physical alterations occur that may have beneficial or detrimental effects on feed components, gastrointestinal development and subsequent performance (Abdollahi *et al.*, 2013).

Longer periods of high temperature after pelleting have been used by feed manufacturers to control the spread of pathogenic microorganisms, especially African swine fever through feed (Wang *et al.*, 2022b). Pelleting further significantly also reduces dustiness of feed while enhancing flow characteristics in augers and bins (Svihus *et al.*, 2004).

Pelleting is a harsh process that degrades vitamins by damaging their structure and the recovery of vitamin A, vitamin E, vitamin B_2, and vitamin B_6 significantly decreased after pelleting and long-term high-temperature stabilization, with the high-temperature stabilization process having the most significant influence (Wang *et al.*, 2022b).

2.5.2 Chemical processing

Chemical treatments are based on the use of substances that have the capacity of altering molecular structures through chemical interactions (Avilés-Gaxiola *et al.*, 2018). The use of acids and bases in combination with physical treatments has been investigated, but the major disadvantage of these processes is that they may result in final products with chemical residues (Soetan & Oyewole, 2009). Chemical processing has been investigated as a method to disrupt the disulfide bonds that gives structure and stability to the trypsin inhibitor tertiary structure (Avilés-Gaxiola *et al.,* 2018)

2.5.2.1 Acids and bases

The use of sodium hydroxide (NaOH), ammonium hydroxide (NH_4OH), and sodium bicarbonate ($NaHCO_3$) during thermal treatment of soybeans has been proposed since extremely high or low pH levels promote loss of enzyme activity due to unfavourable electrostatic interactions between amino acid residues which cause conformational changes in the active site (Avilés-Gaxiola *et al*., 2018). Under acidic conditions, heat inactivation of proteins is not effective and this is utilized during certain forms of chemical processing such as for protein extraction (Accardo *et al*., 2022). The use of alkalis during food processing can cause racemization (the conversion of L-amino acids to D amino acids) that reduces the digestibility of protein between 2-7%, particularly affecting cysteine and aspartic acid (Arora *et al*., 2022).

Sodium hydroxide was specifically emphasised by Van Zyl (2017), for the treatment of starches in maize, however this study focused on nutrition for adult ruminants. The treatment of feed with NaOH has a long research history and Archibald (1924) reported on an improvement in starch digestibility for ruminants when treating cereal grain with NaOH.

Beef cattle are often fed high-concentrate diets to achieve high growth rates, and this practice is strongly associated with metabolic disorders (Nagaraja & Titgemeyer, 2007). Mild acid treatment of grains in high-concentrate diets with 1% hydrochloric acid followed by neutralization with sodium bicarbonate might modify rumen fermentation patterns and microbiota, thereby decreasing the negative effects of high-concentrate diets (Liu *et al.,* 2020).

2.5.2.2 Ammonia / Urea

Ammonia (NH_3) is used during ensiling to add non-protein nitrogen and to increase the concentrations of lactic and acetic acids, decrease proteolysis and improve the aerobic stability (Kung Junior *et al.*, 2000). Ruminants obtain their amino acids from two sources namely ruminally undegraded protein (RUP) and microbial protein synthesized in the rumen (Niazifar, 2024). Urea has been used in ruminant diets for more than 100 years as an additional nitrogen source for the bacteria in the rumen. Its use in dairy cattle diets has fluctuated with protein and urea prices. In a number of studies, the diets were not isocaloric when urea was added, and intake reduction occurred because of high dietary levels of urea (Kertz, 2010). The result for beef cattle, sheep and feedlot lamb is more promising with increased dry matter and crude protein intake and improved digestibility parameters, such as dry matter digestibility and crude protein digestibility reported (Duff *et al*., 2003; Saro *et al*., 2019; Wahyono *et al*., 2022). Urea is hydrolysed by ureases secreted by rumen bacteria to produce ammonia which is used for microbial protein synthesis and the original *in vitro* work of Satter & Slyter (1974), suggested 50 mg/l ammonia-N as the minimum level to avoid constraining microbial protein synthesis. Later *in vivo* studies reported higher optimal levels and Odle & Schafer (1987) reported that the optimal ammonia-N for degradation of barley was 125 mg/l and for of maize 61 mg/l. Urea supplemented in any ruminant feeding system is rapidly degraded to ammonia in the rumen. If urea hydrolysis exceeds the concomitant rate of carbohydrate fermentation it can contribute to less efficient microbial capture of available N (Boucher *et al*., 2007; Ceconi *et al*., 2015; Oliveira *et al*., 2020). The bacterial composition changes in response to total ammonia nitrogen concentrations. High total ammonia nitrogen increases gram-positive *Firmicutes* and *Actinobacteria* but reduces gram-negative *Fibrobacteres* and *Spirochaetes* (Shen *et al*., 2023). As a general guideline a maximum intake of 1% of dietary dry matter intake per animal is recommended where urea-N should contribute a maximum of 30% of total daily N intake (Kertz, 2010; Vargas *et al*., 2024).

Excess ammonia from urea hydrolysis and other N containing compounds are absorbed and transported to the liver (Abdoun *et al*., 2006). Here, ammonia is used for endogenous urea synthesis, which is recycled through the ruminal wall and salivary secretion. This process plays a vital role in N utilization and metabolism in ruminants (Long *et al*., 2004; Reynolds & Kristensen, 2008; Wang *et al*., 2011; Zhou *et al*., 2017). Normally, the liver can detoxify ammonia into urea efficiently by synthesizing urea which is 40 times less toxic than ammonia in the hepatocytes through the Krebs & Henseleit cycle, but at higher concentrations in the blood, ammonia will overwhelm hepatocytes capacity for detoxification. This causes ammonia toxicity, leading to elevated levels of ammonia into the blood (Antonelli *et al*., 2007).

2.5.2.3 Enzymes

The wide variety of enzymes with different characteristics allows for many processing applications (Córdova *et al*., 2022). Although the concept of grain processing with the addition of exogenous enzymes is not new, almost 70 years ago Hastings (1946) reported on the application of exogenous amylase in poultry diets. This concept is still used to enhance digestibility of starch in monogastric species (1996; Rowe *et al*., 1999; Dehghan-Banadaky *et al*., 2007; Cowieson *et al*., 2019) and fibre in ruminants (Useni, 2011). The animal feed industry uses enzymes that degrade crude fibre, starch, proteins, and phytates (Peng *et al*., 2023).

Rising economic pressure enhances the requirement for improved exploitation of low-quality feedstuffs (Ojha *et al*., 2019). The utilization of feed by monogastric animals is not complete and the supplementation of feed with enzymes enhances the nutritive value, thereby increasing the effectiveness of digestion (Alagawany *et al*., 2018). The addition of exogenous enzymes has been shown to feed help break down components such as fibre and phytate that occur naturally in various feed ingredients (Useni, 2011; Bedford & Apajalahti 2022). The presence of these factors may result in decreased meat or egg production and lower feed efficiency, while also contributing to increased digestive disturbances (Sureshkumar *et al*., 2023; Valente Junior *et al*., 2024). Ravindran (2013), reports that exogenous enzymes are mainly added to enhance the accessibility of nutrients from feed ingredients.

The effects of enzymes used in animal feed processing can be attributed to any of the following mechanisms (Bedford & Cowieson, 2012; Ravindran, 2013; Ojha *et al*., 2019):

- The cleaving of bonds or components that cannot be hydrolysed by endogenous enzymes.
- Degradation of anti-nutritional factors that reduce digestibility and increase the viscosity of feed.
- The rupturing of cell wall and releasing of nutrients attached to the cell wall.
- Improvement of nutrient utilization and reducing animal excreta/waste.
- A reduction in the secretions and the loss of endogenous proteins in the intestine, reducing maintenance requirements.
- Increasing insufficient or absent digestive enzymes in the digestive tract, resulting in better digestion, especially in young animals with immature digestive systems.

2.5.3 Biological processing

Biological processing is also referred to as microbial processing (Amit *et al*., 2017), although the term is not always inclusive enough as processes such as germination is often included in this category.

2.5.3.1 Fermentation

Fermentation is an anaerobic and catabolic process where complex molecules are broken down into less complex ones by microorganisms (Sharma *et al.*, 2020b; Buckel, 2021). Bacterial and yeast fermentation involves proteolytic activity that increases amino acid bioavailability by degrading unwanted substances, such as proteinase inhibitors (Avilés-Gaxiola et al., 2018; Xiang *et al.*, 2019). Fermented feed is utilized to promote the digestion and absorption of nutrients in animal diets while enhancing the host's immune system and overall health (Zhu *et al.*, 2023). Fermented feed has been shown to increase the efficiency of nutrient digestion and absorption in livestock, thereby reducing waste production (Lee *et al.*, 2023). Fermentation also contributes to the elimination of pathogenic microorganisms that may be present in the feed (Niba *et al.*, 2009; Lee *et al.*, 2023). The effects of fermented feed, which often contains beneficial microorganisms such as probiotics and yeast, have been extensively studied in various animal species (Pang *et al.*, 2022). In ruminants, research has predominantly focused on the effects of fermented silage, with limited studies on the feeding effects of fermented concentrate. Lee *et al.* (2023) reported that fermenting formulated concentrate can have a positive impact on the growth and health of ruminant livestock.

2.5.3.2 Germination

Germination is a natural catabolic process of plants through which the seed comes out of its latency, using the reserved substances in the cotyledon for embryo development and growth (Sangronis & Machado, 2007). As a processing method germination commonly forms part of malting, as malting consists of steeping, germination, kilning and roasting. Germination has been successfully used as a treatment to reduce anti-nutritional compounds such as trypsin inhibitor. This is achieved as the proteases in the seed digests cellular proteins and releases free amino acids which is subsequently used for seedling autotrophic growth (Budhwar *et al.*, 2020; Gunathunga *et al.*, 2024). Germination is a process of medium efficiency, but it has an additional advantage over other strategies as it increases the concentration and bioavailability of a variety of nutrients and improves specific characteristics of pulses (Hejazi *et al.*, 2016; Gunathunga *et al.*, 2024). If time is not a limiting factor, germination could be combined with other strategies, of at least similar in effectiveness, like gamma irradiation. A combination of both methods may for example help to overcome the losses of vitamin C caused by gamma irradiation (Doblado *et al*., 2007).

Germination terminates dormancy by activating the internal enzymes in grains (Oliveira *et al.*, 2022). It also alters the nutritional profile and morphological structure of grain seeds, ultimately improving the digestibility and bio-accessibility of nutrients. (Chu *et al.*, 2020; Li *et al*., 2022). Germination has also been shown to improve the bioactivities of food components by generating new bioactive compounds such as polyphenols and flavonoids, which accounts for the high antioxidant activity in grain seeds (Xu *et al.*, 2019; Chu *et al.*, 2020). Therefore, germination boosts human health by

providing neuroprotective, anti-cancer, anti-bacterial, anti-diabetic, anti-inflammatory, and cholesterol-lowering properties against many communicable and non-communicable diseases (Chu *et al.*, 2020).

2.6 Weaning shock

Wean shock is commonly characterised by body weight loss due to weaning and occurs due to the abrupt shift from a milk-based diet to a diet of solid feed when calves are weaned (Sweeney *et al.*, 2010). Calves must adapt to a new source of nutrition, which can initially lead to a temporary reduction in dry matter intake. This weight loss is a common and expected outcome of the weaning process but can be managed with proper care and nutrition (Zeleke et. al., 2017). Elevated stress associated with weaning can weaken the immune system of calves, making them more susceptible to diseases and stress-induced health problems, such as respiratory infections and diarrhoea, can occur if not managed properly (Hulbert & Moisá, 2016). Prolonged or severe wean shock can negatively impact on the growth and development of calves. And it can take some time for calves to adjust to their new diet and environment and resume normal growth rates. Adequate nutrition and management practices are therefore essential to ensure that calves rapidly recover their weight and grow optimally (Tang *et al.*, 2022).

When the transition from liquid to solid feed is too abrupt, it can have a significant impact on the digestive tract of the young calf leading to digestive upsets such as diarrhoea and subsequent weight loss. Successful transition between milk replacers is dependent on enzyme activity levels and as enzyme activity is stimulated by the presence of specific nutrients, the gradual introduction of the new nutrients in the starter meal can help to avoid diarrhoea and potential mortalities (Dennis *et al.*, 2018; Klopp *et al.*, 2019; Zhang *et al.*, 2019). When calves are weaned, the transition is from liquid diets flowing to the abomasum whereas solid feed will be going into the rumen (Dias *et al.*, 2017). This implies a transition from mainly relying on enzymatic digestion to the fermentative breakdown of feed in the rumen (Ørskov, 1972; Kaba *et al.*, 2018; Diao *et al.*, 2019).

A common misperception is that the rumen can be developed by over-feeding, causing the milk replacers to overflow into the rumen. While this overflow can provide additional nutrients to the developing rumen, it may disrupt the normal digestive process and compromise the efficiency of nutrient utilization. (Hill *et al.*, 2016). For the rumen to provide sufficient nutrients to the ruminant it must be adequately developed and contain the required microbial population to be able to support fermentation on the required scale (Dias *et al.*, 2017; Zhang *et al.*, 2021b).

Rumen development is of crucial importance during the early life stages of the calf as it influences the overall health and future productivity of the animal (Malmuthuge *et al.*, 2019; Zhang *et al.*, 2019; Li *et al.*, 2023). The introduction of starter feed is a pivotal factor in stimulating rumen development. High-quality starter feeds, typically containing a mix of grains and fibrous ingredients, play a crucial

role in promoting rumination and microbial activity in the developing rumen (Cammack *et al.*, 2018). From day 7 microbial activity in the rumen significantly increases with the introduction of solid feed (Dias *et al.*, 2017). Rumen development in calves is a multifaceted process that begins shortly after birth. Initially, the undeveloped rumen relies on the absorption of volatile fatty acids (VFAs) produced during fermentation. As the calf consumes starter feed, the rumen undergoes morphological changes, such as papillae development, which enhances its capacity for nutrient absorption (Baldwin *et al.*, 2004; Lesmeister & Heinrichs, 2004). Practical considerations for optimizing rumen development include the strategic introduction of starter feed, monitoring calf growth, and adjusting feeding practices based on individual needs. Proper nutrition and management during the early stages are critical for establishing a healthy and functional rumen (Steele *et al.*, 2016; Diao *et al.*, 2019).

Calves raised by well-nourished dams often receive milk with a balanced nutrient profile, promoting higher feed intake after weaning due to enhanced palatability and nutritional content (Kertz *et al.*, 2017). Weaning calves will lead to weight loss if the rumen cannot sufficiently support fermentation as at this stage as the rumen becomes the main digestive system through which the calf acquires nutrients (Carballo *et al.*, 2019; Diao *et al.*, 2019; Li *et al.*, 2023). Although other external factors such as environmental changes may play a role, if managed correctly the only significant factor is the change in diet (Cholewińska *et al.*, 2021). It is advised that weaning should be a gradual process, and it should be ensured that starter feed of a high quality is available while calves are still suckling (Franklin *et al.*, 2003; Bittar *et al.*, 2020).

Contrary to starter feed, milk has a limited role in rumen development. While milk provides essential nutrients it does not contribute significantly to the physical development of the rumen (Diao *et al.*, 2019). Glucose, along with fatty acids, is considered a key energy substrate for the immature rumen epithelial tissue, especially before active fermentation begins in the rumen and these nutrients are absorbed by the small intestine (Pokhrel & Jiang, 2024). The early stages of rumen development are characterized by a gradual transition from a reliance on milk to the incorporation of solid feeds. If a milk replacer induces starter feed intake a milk replacer can contribute indirectly to optimising rumen development in the early stages of a calf's life (Baldwin *et al.*, 2004; Khan *et al.*, 2016; Pokhrel & Jiang, 2024). Feed intake is a critical aspect of early calf development, influencing growth, health, and overall productivity. Early exposure to solid feed plays an important role in stimulating starter feed intake in suckling calves. Calves that are introduced to starter feeds early in life tend to have a smoother transition to solid diets, establishing feeding behaviour and preferences (Khan *et al.*, 2011). The nutrient density of milk, particularly the protein and fat content, however, influences calf satiety and feed intake (Araujo *et al.*, 2014). Higher dietary fat intakes from liquid feed reduced hunger-related behaviour during the weaning transition in *ad libitum* fed calves (Echeverry-Munera *et al.*, 2021). Increasing milk or milk replacer allowances to more than 0.8 kg/d reduces starter feed intake (Cowles *et al.*, 2006; Hill *et al.*, 2010; Davis Rincker *et al.*, 2011). This delayed rumen development

(Terré *et al.*, 2007; Suárez-Mena *et al.*, 2011), and postweaning average daily gain (ADG) is associated with depressed feed efficiency and digestibility after weaning (Jasper & Weary, 2002; Cowles *et al.*, 2006; Hill *et al.*, 2007). Therefore, when offering increased amounts of milk or milk replacer, it is especially important to stimulate solid feed consumption to avoid a decline in performance and impairments of health after weaning (Araujo *et al.*, 2014).

The beneficial effect of a high-protein intake seems to be due to increased diet-induced thermogenesis (DIT) (Mikkelsen *et al.*, 2000), increased satiety (Weigle *et al.*, 2005; Astrup, 2005) and decreased hunger (Skov *et al.*, 1999). It has been hypothesized that αlac has a beneficial effect on satiety owing to a high content of essential amino acids such as leucine, lysine, and tryptophan (Chatterton *et al.*, 2006; Hursel *et al.*, 2010; Bendtsen *et al.*, 2013). Tryptophan is a precursor of the neurotransmitter serotonin, which acts as an anorexigenic signal in the brain stimulating satiety (Jenkins *et al.,* 2016). Leucine and lysine are ketogenic amino acids, and it has been shown that appetite decreases under ketogenic conditions (Johnstone *et al.*, 2008).

High-quality milk with appropriate nutrient levels encourages consistent and adequate feeding behaviour in suckling calves (Jenkins *et al.*, 2012; Soberon *et al.*, 2012). The palatability and texture of starter feeds significantly influence feed intake (Terré *et al.*, 2016; Pazoki *et al.*, 2017). Calves show a preference for textured feeds with pleasant taste, promoting active feeding behaviour (Nedelkov *et al.*, 2019). The incorporation of palatable starter feeds encourages voluntary intake and helps meet nutritional requirements (Miller-Cushon *et al.*, 2014; Nedelkov *et al.*, 2019).

Starch provides a source of readily available energy, contributing to the overall energy density of the diet (Hua *et al.*, 2022). Starch enhances the energy content of the diet, promoting increased appetite and overall nutrient consumption. However, the optimal level of starch in milk must be carefully balanced to avoid digestive upsets and ensure calf health (Huber *et al.*, 1968; Lesmeister *et al.*, 2004). Feed intake in suckling calves is influenced by a combination of factors, including maternal nutrition, early exposure to solid feed, nutrient density of milk, and the starch content in the milk replacer (Hepola *et al.*, 2007; Asheim *et al.*, 2016). While starch in milk can have a positive impact on feed intake by enhancing energy density, careful consideration of the optimal starch level is essential to prevent potential digestive disturbances (Gilbert *et al.*, 2015). Balancing these factors ensures a successful transition to solid diets, supporting the growth and well-being of suckling calves.

2.7 Calf body measurements

Body weight of calves is often used to determine the financial value of an animal and to evaluate the efficacy of management and feeding regimes. Birth weight of calves is used as indicator of foetal growth, particularly during the last trimester of gestation as approximately 70% of birth weight is added during this period (Micke *et al.*, 2010). Birth weight data can assist dairies to make better

decisions about calf rearing and pregnant cow nutrition management. Live weight gain is used for managing feeding regimes (Dingwell *et al.*, 2006; Curtis *et al.*, 2018) while estimated body weight is used to determine the dosage of medications, including antibiotics and anthelmintics (Enevoldsen & Kristensen, 1997; Dingwell *et al.*, 2006; Machila *et al.*, 2008; van Dijk *et al.*, 2015). These applications can help to improve the welfare of livestock and the economic position of subsistence farmers.

The most accurate way to determine the body weight of a calf is to use an electronic scale. The use of an electronic scale however comes at a cost implication to farmers, and this is often beyond the means of farmers and can be time-consuming if there is a lack of infrastructure. For this reason, it might not be the preferred method even on farms that do have access to an electronic scale (Heinrichs *et al.*, 1992; Dingwell *et al.*, 2006).

In response to the above reasons, methods have been developed to estimate the weight of a calf without the use of an electronic scale. One of these methods is the use of a measuring tape (Dingwell *et al.*, 2006). The use of a measuring tape allows the farmer to estimate the weight of cattle without being restricted by the lack of portability of a scale and although there is handling involved, it's easy to learn and apply. The measuring system exploit the high relationship between girth circumference of calves and their weight (Wangchuk *et al*., 2017).

Currently there is no single standard with regards to measuring tapes and the predicted weight when using different tapes vary considerably (Heinrichs *et al*., 2007). This result is influenced by several factors and the specific algorithm fitted to the data used when developing the specific measuring tape (Ruchay *et al*., 2022). Commonly used tapes include tapes that are used to measure the girth and hoof circumference, and studies have been conducted to compare the two methods as well as comparing the predicted weights using these methods with scale weight (Sharpe & Heins, 2023). The girth tape is marked in centimetres (or another measuring unit) and predicts the corresponding body weight for each increment. The predicted weight is derived from a model developed by measuring the weight and girth of many cattle. The girth tape measures the chest circumference of the body behind the front shoulder. The hoof girth tape measures the circumference of a specific hoof of the calf and the weight is then predicted using this parameter.

Heinrichs & Hargrove (1987) measured 5,723 heifers on commercial farms in Pennsylvania and reported a good correlation between heart girth circumference and body weight. This formed the basis of the heart girth tape, used for estimating the weight of calves. It should be noted that the original population of animals measured to determine the correlation between body circumference and weight were all heifers (Heinrichs & Hargrove, 1987).

Age of calves appears to have an influence on the accuracy of prediction when using a heart girth tape. Dingwell *et al.* (2006) reported a significant difference between scale weight and heart girth tape in calves younger than three months and that heart girth tape measurement was significantly lower than the scale measurements. It is important to note that this period is mostly during the pre-weaned phase and that the diet during this period largely consists of milk and milk replacers. Dingwell

et al. (2006) further recommends that the study of the heart girth tape for predicting the weight of very young Holstein calves should be investigated further.

Breed is presumed to influence the accuracy of predictions when using the heart girth tape and some studies suggest that better results are obtained for certain breeds when using specific tapes (Conan *et al*., 2018). Sharp & Heins (2023) report that accuracy has been confirmed to a larger degree in some purebred dairy breeds, but that the use of heart girth tapes and hoof-circumference tapes may not be accurate in estimating the weight of crossbred calves.

Sex does not appear to have a specific influence on accuracy of weight predictions when using heart girth tapes, although according to Odadi (2018), these measurements were taken in sexually immature animals, and this may not hold true for older animals.

The location of a specific population seems to influence results as Heinrichs *et al*. (2007) and Milla & Mahjoub (2013) reported that the use of a girth tape not created specifically for that population of cattle needed to be transformed to improve prediction accuracy. This may be because of location, differences in livestock management, genetics and environmental conditions on girth circumference (Magnabosco *et al*., 2008). This suggests that specific herd factors relating to nutritional management, body condition, or genetics of conformation may play an important role in accuracy of predictions.

The season and therefore climate may also influence the accuracy of the heart girth tape as body condition is often influenced by the season. MacDonald *et al*. (2014) reported that approximately 70% of the cattle that they measured were in medium condition during the dry season within a one percent increase for the wet season. There was a decrease in the percentage of fat cattle in the wet season from 24% in the dry season to 21% in wet. The accuracy of prediction varied between the summer and winter season, and MacDonald *et al*. (2014) therefore suggested that separate tapes should be developed for the different seasons.

Although handling error is separate from the other external factures, it also has a direct influence on the accuracy of weight predictions when using the heart girth tape (Wood *et al*., 2015). Heart girth measurements may be difficult to perform consistently, as a change in the position of the animal during measurement or the tightness of the tape can all affect the results (Wood *et al*., 2015).

It is therefore clear that multiple factors can affect the accuracy when using the girth circumference method to estimate body weight, but the accuracy of prediction can be improved by taking location, breed, gender, body condition, age and the breed used to calibrate the tape into account.

2.8 Conclusion

The pre-ruminate digestive system is complex as it involves not only enzymatic digestion, but also the development of the rumen and therefore the establishment a microbial population in the rumen. This population is responsible for the microbial fermentation of feed. As milk replacers are composed

of different fats, proteins and carbohydrates, it further complicates the identification of the specific effects of these components in the digestive system. Different processing methods of the raw materials also influence the effect of these milk replacers on the pre-ruminant digestive tract. There is limited information on increased carbohydrates content in milk replacers for neonatal calves, providing research opportunity. Although fermentation as a processing method has been well researched, specific fermentation processing applied to producing raw materials for milk replacers has to be investigated in more depth. When investigating the effect of specific raw materials used in milk replacers on the pre-ruminant digestive system the most effective way is to use a live model. The strive for alternative and more cost-effective calf milk replacers for effective production and potential reduction of calf morbidity seems viable and more research is required in this regard.

2.9 References

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Chapter 3
The effect of milk replacers containing fermented plant protein and high carbohydrate content on the growth performance of Holstein bull calves

3.1 Abstract

This study investigated the effects of fermented protein (FP) and increased carbohydrate (HC) levels in milk replacers on calf growth performance. Fermented protein was developed as an alternative raw material to enhance the amino acid composition of plant-based substrates in milk replacers, with the aim of improving growth outcomes. Two milk replacers were formulated: one containing fermented protein and the other using a conventional protein source (standard protein, SP). To assess the impact of increased carbohydrate levels, two additional milk replacers were formulated, one with fermented protein and one without, both containing 20% pre-cooked maize.

A total of 32 Holstein bull calves were randomly assigned to four treatments (n = 8) in a 77-day trial, which was divided into two phases: Phase 1 (P1, days 0–63), during which milk replacers were fed, and Phase 2 (P2, a two-week post-weaning period) to evaluate the occurrence of weaning shock. Milk replacer intake was controlled, while starter meal intake was offered *ad libitum*, and all intakes and refusals were recorded.

Growth performance parameters, including weight gain, dry matter intake (DMI), average daily gain (ADG), DMI as a percentage of body weight (DMI/BW %), and feed conversion ratio (FCR), were determined and analysed using factorial ANOVA. The only significant interaction observed was for FCR ($P = 0.036$) during the total trial period. Except for controlled milk replacer intake and P2 DMI/BW% and P2 FCR, fermented protein containing milk replacers generally resulted in poorer performance ($P < 0.05$) compared to SP milk replacers. This was attributed to higher levels of trypsin inhibitors in the fermented protein, which negatively impacted performance.

The high carbohydrate milk replacers promoted greater starter meal intake during all phases ($P < 0.05$). Based on these results, it is recommended that milk replacers with higher carbohydrate content can be beneficial to stimulate starter meal intake, while the use of fermented protein as a raw material should only be considered after the fermentation process is better optimized to reduce trypsin inhibitor levels to below 4 mg/g protein.

3.2 Introduction

At birth, the calf's rumen is undeveloped and microbial fermentation do not play a significant role in the digestive process. Young calves are therefore referred to as pre-ruminants (Porter, 1969). During

this phase, the abomasum and duodenum are the most important compartments where enzymatic digestion takes place and milk is directly shunted into the abomasum, bypassing the rumen due to the reflex closure of the oesophageal groove (Ørskov, 1972).

The fact that different milk replacers influence growth rate and the incidence of diarrhoea in calves is well documented (Amado *et al.,* 2019; Johnson *et al.,* 2019). This effect differs depending on the specific rearing conditions. Different protein- and energy sources impacts on the performance of calves (Brown *et al.*, 2005). The biological and economic viability of a specific feeding regime depends on growth rate, feed conversion, cost of the milk replacer and the incidence of growth insults caused by factors such as diarrhoea (Bach *et al.,* 2013; Hu *et al.,* 2019).

3.2.1 Protein

Proteins are organic compounds with amino acids as building blocks (Kitadai & Maruyama, 2018). These amino acids are linked by peptide bonds to form a chain (Jacob & Monod, 1961). Polypeptides composed of more than fifty amino acids are classified as proteins (Numata, 2020). Proteins are important as a structural component in animal tissues as well as in biochemical, immunological, transportation and hormonal processes (Morris *et al.,* 2022). The quality of proteins is important in formulation of milk replacers and is defined as the ability of a specific protein to provide the necessary amino acids in the required ratio for optimal growth and development of the neonatal calf (Katz *et al.,* 2019; Schubert *et al.,* 2022). For pre-ruminants the protein in milk has a high apparent digestibility and the absorbed amino acids are efficiently utilized for protein synthesis (Huang *et al.,* 2015).

Enzymatic digestion of milk in suckling ruminants starts with the clotting of milk in the abomasum. This is affected by a combination of chymosin, and hydrochloric acid secreted in the abomasum (Andrén, 2011; Miyazaki *et al.,* 2019). The clot slows down the rate of passage through the abomasum of calves (Cruywagen *et al.,* 1990). This results in more time in the abomasum and a more controlled flow of chyme through the pyloric valve, improving digestion and absorption in the small intestine (Petit *et al.,* 1987; Longenbach & Heinrichs, 1998; Andrén, 2011).

Protein digestion involves the denaturing to expose peptide bonds allowing hydrolyzation of the molecule, and the release of free amino acids (Rivera Del Rio *et al.*, 2021). Protein-digesting enzymes in the abomasum includes endopeptidase or exopeptidase (Kurz & Seifert, 2021). Endopeptidase cleaves the peptide bonds while exopeptidase cleaves amino acids off at the terminal end of the protein molecule (Van der Velden & Hulsmann, 1999; López-Otín & Bond, 2008).

The protein requirement of cattle depends on age, physiological stage (e.g., pregnancy or lactation), pathological status and the quality of the protein supplied (NRC, 2021). It is further influenced by the extent to which different feedstuffs supply essential nutrients needed for efficient, economical and sustainable production (Beever, 1996). The basic requirement of dietary protein is thus to supply adequate amounts of required amino acids in the correct ratio (NRC, 2021). According to Ertl *et al.*

(2016), the amino acid profile of the protein in the diet, the essential amino acids as well as the concentration of the limiting amino acids and the digestibility and physiological utilization of amino acids determine the value of a protein source. Protein quality can be defined as the ability of the ingested protein to meet the requirements for all the dietary indispensable amino acids in an animal, which can be measured by the Digestible Indispensable Amino Acid Score (DIAAS; Wolfe *et al.,* 2016). The bioavailability of an amino acid can be defined as the amino acid in a form suitable for digestion, absorption and utilization and can only be estimated using animal growth bioassays with live animals (Jahan-Mihan *et al.,* 2011). The origin of protein, such as animal versus plant protein and feed processing methods can also affect protein digestibility, influencing bioavailability. Animal protein compared to plant protein is considered to contain a better balance between essential amino acids and nonessential amino acids (Ertl *et al.,* 2016). Plant proteins, such as soybean meal, normally also contain antinutritional factors (e.g., trypsin inhibitor) that can impair protein digestibility and availability of amino acids (Jahan-Mihan *et al.,* 2011). It has been shown that feed processing methods including heating, re-roasting and extrusion can inhibit antinutritional factors like trypsin inhibitor in soybean meal (Akande & Fabiyi, 2010). However, overheating makes amino acids unavailable due to a complex reaction with sugars (browning or Maillard reaction; El-shemy *et al.,* 2000).

As the fermentation process generally improves the digestibility of proteins (Sánchez-García *et al.,* 2024), it was hypothesised that fermented plant protein could improve growth performance of neonates compared to that of standard plant protein when used in milk replacers. During the fermentation process, microorganisms release peptides and amino acids from complex storage proteins, making the proteins more soluble and digestible (El-Hag *et al.,* 2002; Alka *et al.,* 2012; Ali *et al.,* 2003; Pranoto *et al.,* 2013). Thus, bacterial fermentation increases available lysine concentration and nutritive value (Hamad & Fields, 1979; Wakil & Onilude, 2009). Bacterial and yeast fermentations involving proteolytic activity are expected to increase the biological availability of essential amino acids and degrade carbohydrates and other unwanted substances (Adeyemo & Onilude, 2013). Fermentation may also increase digestibility by reducing the levels of non-nutritive compounds that inhibit digestive enzymes (e.g. trypsin and chymotrypsin inhibitors) and promote protein crosslinking (e.g. phenolic and tannin compounds). Protein matrixes are also partially degraded by microbial proteases, releasing some of the proteins from the matrix, thereby improving mineral bioavailability (Shekib, 1994; Reyes-Moreno *et al.,* 2004; Chandra-Hioe *et al.,* 2016) and phytic acid that binds minerals is reduced, making them free and more available (Lopez *et al.,* 1983; Hemalatha *et al.,* 2007). Tannins bind minerals and reduce their bioavailability (Emambux & Taylor, 2003), but fermentation loosens the complex matrix that embeds minerals (Nkhata *et al.*, 2018). Both phytase and α-amylase loosens the matrix bonds by degrading phytate and starch, respectively (Nkhata *et al.,* 2018). Moreover, some fermenting microorganisms degrade fibre by loosening the food matrix (Liang *et al.,* 2008). Fermentation further improves mineral bioavailability, as microbial metabolism generates organic acids, which then form soluble complexes with mineral compounds

preventing the formation of insoluble mineral-phytate complexes (Hemalatha *et al.,* 2007; Çabuk *et al.,* 2018). The fermentation process also lowers the pH, and this increases iron absorption due to conversion of low absorbable ferrous iron to the readily absorbable, ferric iron (Leenhardt *et al.,* 2005; Hemalatha *et al.,* 2007; Nkhata *et al.,* 2018).

3.2.2 Carbohydrates

Although enzymatic digestion starts in the abomasum of the pre-ruminant, no significant carbohydrate digestion occurs here and enzymatic carbohydrate digestion starts in the small intestine (Vargas-Rodriguez *et al.,* 2014; Hua *et al.,* 2022).

Lactose, commonly referred to as milk sugar, is the most important carbohydrate present in milk (Gambelli, 2017). Lactose must be hydrolysed before it can be absorbed (Dollar & Porter, 1957; Blaxter, 1988; Roy, 1990). Beta-galactosidase is responsible for lactose hydrolyses in the small intestine (Juers *et al.,* 2012). The secretion of β-galactosidase increases shortly after birth and declines with age with a tenfold higher secretion in 8-week-old calves compared to adult ruminants (Coombe & Siddons, 1973; Toofanian *et al.,* 1973; Huërou-Luron, 2002). Pre-ruminants can readily utilize glucose, galactose and lactose, but has a limited ability to utilize maltose and starches and is unable to utilise sucrose (Dollar & Porter, 1957; Walker, 1959; Estévez *et al.,* 2014).

The building blocks of starch are glucose molecules and if starch is broken down to oligosaccharides and disaccharides, maltotriose and maltose is formed (Salema-Oom *et al.*, 2005; Damager *et al.,* 2010). Maltase and isomaltase activity therefore directly influence the rate at which starch is broken down and the extent to which it can be utilized by neonatal and young calves. During the first four weeks of life, maltase and isomaltase activity increase, reaching adult levels (Le Huerou *et al.,* 1992). In the adult ruminant isomaltase activity is half that of maltase activity (Coombe & Siddons, 1973; Toofanian *et al.,* 1973; Le Huerou *et al.,* 1992).

The rate of starch digestion is largely determined by physicochemical characteristics such as granule size, degree of crystallinity, the amylose to amylopectin ratio and the presence of other compounds (Parada & Aguilera, 2011). Starch is also broken down in the large intestine by microbial activity (Serena *et al.,* 2008). Excessive intake of starch and any sugar, even lactose and glucose, can cause diarrhoea in pre-ruminant calves. There is agreement in literature that the amount of glucose that can be absorbed from the small intestine of the pre-ruminant is limited (Velu *et al.,* 1960; Ballard & Oliver, 1965; Trotta *et al.,* 2022). As higher starch inclusion can be used to increase the carbohydrates in high carbohydrate milk replacers, maltase and isomaltase activity levels are important to determine the optimal age at which the transition can be made from a standard milk replacer to a high carbohydrate milk replacer. Maltose activity gradually increase and in two-week-old calves the enzyme activity reaches half the level of activity of adult cattle (Krehbiel *et al.,* 1996;

Le Huerou *et al.,* 1992). At this age the enzyme activity is at a level where milk replacers with a high starch content can be fully utilized by suckling calves.

3.2.3 Aims

This study aimed to compare a conventional commercial milk replacer with a formulated milk replacer containing 20% of a specific fermented plant protein as a raw material. These milk replacers were further compared with a commercial high carbohydrate milk replacer and a high carbohydrate milk replacer formulated to contain 20% of the same fermented plant protein. This was done in a calf growth trial, using a 2x2 factorial design with the aim to evaluate the effect of the different milk replacers on growth parameters, dry matter intake and feed efficiency of neonatal calves. After weaning the carry over effect of the different milk replacers were evaluated, using the same parameters. This included determining the presence and extent of wean shock.

3.3 Materials and methods

Ethical clearance was obtained from the UFS Animal Research Ethics Committee for all trial related practices (UFS-AED2023/0051).

A calf rearing trial with 32 Holstein bull calves fed four different milk replacers as treatments was executed. This took place at the calf rearing unit of Livestock Wellness in George who provided the different milk replacers for the trial in collaboration with Nandrea Health Products. The four experimental groups for the trial were:

- The first group, A, was fed Biomel®, a commercial milk replacer and this group served as the control.
- The second group, B, was fed a milk replacer containing fermented plant protein.
- The third group, C, was fed Kalfpap®, a high carbohydrate milk replacer.
- The fourth group, D, was fed a high carbohydrate milk replacer containing fermented plant protein.

The fermentation of the plant medium to produce the fermented protein (FP) was conducted at Nandrea Health Products in Oudtshoorn. In a previous study (Cordy, 2024), the nutrient value of fermented protein was determined by performing a proximate, fatty acid and amino acid analysis (Table 3.1). The fermented protein was used as a raw material and these values were used during the formulation of iso-caloric and iso-nitrogenous milk replacers for the respective treatments (Table 3.4).

Table 3.1 The proximate, amino acid and fatty acid composition of a 1000-liter fermentation of soybean over a seven-day fermentation period at 40°C (Cordy, 2024).

Proximate (g/kg)		**Fatty acid (mg/g)**		**Amino acid (%Lys)**	
Dry matter	864.25	C10	0.76	Lys	100
Crude protein	339.12	C12	0.85	Arg	135
Carbohydrates	79.70	C14	2.79	Ser	96
Moisture	135.75	C15	0.47	Gly	86
Ash	95.36	C16	32.58	Asp	214
Acid detergent fibre	125.04	C16:1	0.59	Glu	323
Neutral detergent fibre	114.95	C17	0.55	Thr	80
Ether extract	235.11	C18	14.63	Ala	81
		C18:1 (trans)	0.18	Pro	103
		C18:1 (cis)	42.16	His	56
		C18:2 (cis)	108.47	Tyr	81
		C20	1.11	Met	39
		C18:3n6	0.32	Val	97
		C20:1	0.39	ILe	90
		C18:3n3	19.79	Leu	154
		C21	0.14	Phe	111
		C20:2	0.12		
		C22	1.08		
		C20:4n6	0.10		
		C23	0.12		
		C24	0.40		

Appendix A, Table A.1 provides a detailed description of the trial and feeding procedure. During the trial a regime of 3 feedings of 2 L milk replacer per day was used. On day 1 of the trial, the calves received 1 L per feeding, and this was increased in steps of 200 ml per feeding per day until 2 L per feeding was reached. At the end of the trial the milk replacers were decreased gradually to 1 L and 2 feedings per day. After weaning the calves were kept in the unit for a further 2 weeks where only starter meal was fed *ad libitum*. Any milk replacer refusals were noted, and total milk intake was compounded on a weekly basis. During the calf rearing trial, a standard starter meal (Table 3.4) was used, and the calves had *ad lib* access to it throughout the trial. A cumulative weekly starter intake was determined and reported by weighing feed offered and refusals.

The trial data was grouped according to the different periods as depicted in Figure 3.1. Phase 1 (P1) refers to the period during which the calves were fed milk replacer and was from day 0 – 63. Day 63 was also the day on which the calves were weaned. Phase 2 (P2) was the two-week observation period after weaning, starting on day 64 and terminating on day 77. This phase was used to determine the level of wean shock. The total phase (TP) started on day 0 and terminated on day 77.

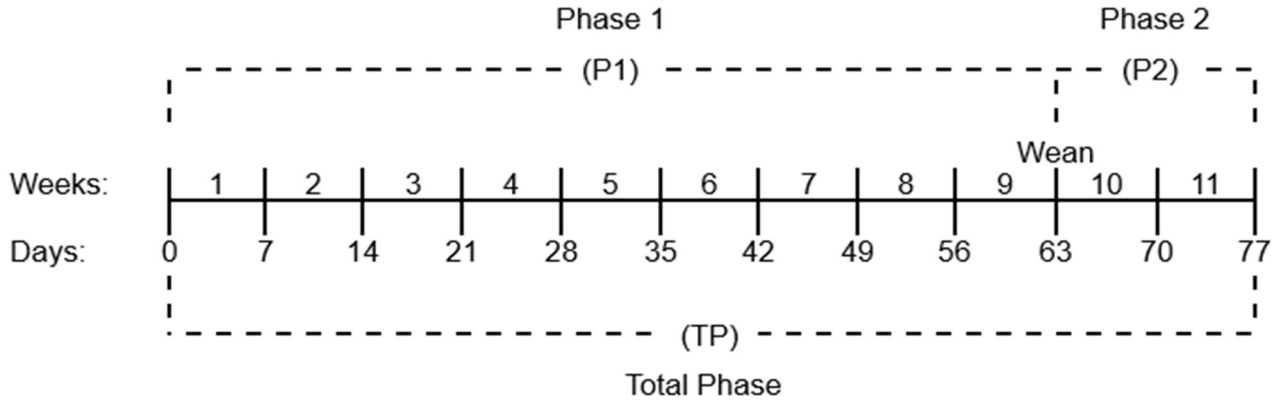

Figure 3.1 A line diagram of the time periods used in the trial. The direct influence of the different milk replacers was investigated during P1. Transfer effects such as wean shock were investigated during P2 and TP was used to determine direct and transfer effects.

Thirty-two Holstein bull calves, supplied by a commercial supplier, were marked and numbered with different colour tags and randomly allocated to the respective treatment groups. All the calves received colostrum directly after birth before they were entered into the trial at an age between 3 and 4 days old. Calves treated with high carbohydrate milk replacers were gradually transitioned from standard milk replacer to the high carbohydrate milk replacers. This was done to limit the incidence of diarrhoea due to a change in the diet. It is well documented that when calves are 11 days old the activity levels of starch digestive enzyme such as amylase, maltase and isomaltase should have increased to such an extent that it is possible to successfully introduce the high carbohydrate milk replacers (Miyashige & Yahat, 1980; Harmon, 1993). Harmon (1993) further highlights that the secretion and activity of amylase activity can be manipulated nutritionally. During the current trial this transition started with the inclusion of 25% high carbohydrate milk replacer on day 11 and this was increased in fractions of 25%, reaching 100% on day 14.

A standard vaccination program was followed (Appendix A). Calves were weighed at the same time and day on a weekly basis. Girth circumference was also determined each time the calves were weighed.

All data were analysed according to multiple ANOVA with main effects being carbohydrate inclusion and protein source. As a factorial design (Table 3.2) was used to analyse the data, the interactions were investigated first, as the occurrence of interactions precludes attributing results to specific effects. Where no specific interaction was observed, the difference was attributed to the main effect.

Main effects as well as commercial milk replacers are shown in Table 3.2.

Table 3.2 The experimental design of the trial, with a total of 32 calves (N = 32) allocated to four different treatments (n = 8).

	Standard protein (SP)	**Fermented protein (FP)**
Standard carbohydrate (SC) inclusion levels	n = 8 (Biomel®, V11906) **A**	n = 8 (FP-Biomel) **B**
Higher carbohydrate (HC) inclusion levels	n = 8 (Kalfpap®, V17059) **C**	n = 8 (FP-Kalfpap) **D**

When interactions were observed, data were simultaneously interpreted. For the parameters tested there are therefore three sets of null and alternative hypotheses. For clarity on when the null hypotheses were accepted or rejected, using the P-values, Table 3.3 lists the hypotheses for this factorial design.

Table 3.3 The hypotheses used in the analysis of the data for the growth performance parameters analysed with a multiple (factorial) ANOVA.

	Null and alternative hypotheses	**P-value interpretation**	
Interaction	H_0: An interaction is absent H_A: An interaction is present	P-value ≤ 0.05 {Reject H_0}	Interaction
		P-value > 0.05 {Accept H_0}	No interaction
Protein main effect	H_0: $\mu_{SP} = \mu_{FP}$ H_A: $\mu_{SP} \neq \mu_{FP}$	P-value ≤ 0.05 {Reject H_0}	$\mu_{SP} \neq \mu_{FP}$
		P-value > 0.05 {Accept H_0}	$\mu_{SP} = \mu_{FP}$
Carbohydrate main effect	H_0: $\mu_{SC} = \mu_{HC}$ H_A: $\mu_{SC} \neq \mu_{HC}$	P-value ≤ 0.05 {Reject H_0}	$\mu_{SC} \neq \mu_{HC}$
		P-value > 0.05 {Accept H_0}	$\mu_{SC} = \mu_{HC}$

To verify nutrient values random starter feed and milk replacer samples were taken from different batches throughout the trial and the pooled samples were analysed as shown in Table 3.4. The protein content of the starter feed and milk samples were determined according to standard methods prescribed by AOAC (2002) using the Dumas method and a LECO FP828 (St. Joseph, MI, USA). Since fats in milk replacers cannot be accurately determined by a proximate analysis (ether extract) as is does not efficiently extract emulsified fats (Moneeb *et al.*, 2021), the fat content in the milk replacers was determined by Assurecloud using a gravimetric method (Evers *et al.*, 2000).

Table 3.4 The calculate carbohydrate percentage and proximate analysis (dry matter basis) of the milk replacers and starter meal (SM).

Milk replacer and Starter meal	**Carbohydrate %**	**Nitrogen** (g/kg)	**Crude Protein** (g/kg)	**Crude Fat** (g/kg)	**Metabolizable Energy** (MJ/kg)
A	40.68	33.3	208.1	159.2	12.85
B	40.47	33.2	207.5	155.4	11.99
C	49.57	31.2	195.0	116.2	12.63
D	48.85	32.1	200.6	115.5	12.27
SM		24.7	154.4	3.17	10.57

The crude protein values for the milk replacers ranged between 195.0 g/kg and 208.1 g/kg (Table 3.4) and the value for the starter meal was 154.4 g/kg. The values of the crude fat for the conventional

milk replacer (A and B) ranged between 155.4 and 159.2 g/kg, and for the high carbohydrate milk replacers (C and D) ranged between 115.5 and 116.2 g/kg. The lower crude fat content of the milk replacers containing high carbohydrate can be attributed to the higher carbohydrate content as the different milk replacer were formulated to be iso-caloric. The crude fat content of the starter meal was 3.17 g/kg and this is in line with the general prescription for ruminating cattle (Fiorentini *et al.*, 2015; Bionaz *et al.*, 2020).

3.4 Results and Discussion

The interaction reactions were first investigated as shown in Table 3.5 to determine if there were any result that could be attributed to an interaction effect. The only P-values indicating an interaction was for FCR for TP (P = 0.036; Table 3.5), presented in bold. For all other parameters no interaction effect was observed, and main effects could be used to interpret the data for the different parameters. The data in Table 3.5 is discussed as it helps to elucidate the main effects.

The initial weight of TP and P1 is the weight on commencement (Day 0) of the trial as the starting point for these two periods are the same. As the calves were randomly allocated between treatment groups, the average initial weight for the four treatment groups did not differ. This confirms the efficacy in allocating animals to treatments and excludes the effect of body weight at commencement of the trial. The final weight of P1 and the initial weight of P2 are the same (Day 63) as these phases follow chronologically and P2 started immediately after P1 was completed. Weight on day 63 for treatments A, C and D did not differ, and B and D also did not differ (Table 3.5). It is therefore clear that the different milk replacer treatments did affect weight gain differently during the weaning phase. The final weight of TP and P2 is the same as both ended on day 77 of the trial. Weight on day 77 for treatment groups A and C did not differ and were the highest while A and D also did not differ (Table 3.5). While the average weight of group B was significantly lower ($P < 0.05$) than that of the other treatment groups as differences became more distinct. There was an indication that a transfer effect from the milk replacer treatment to P2 after weaning was present since treatment B already had lower body weight after P1, albeit not differing from that of treatment D (Table 3.5). Although the final weigh is influenced by the milk replacers, starter meal intake might also have been stimulated differently by the different milk replacers, potentially reducing wean shock, especially for the high carbohydrate groups.

Table 3.5 A summary of calf performance parameters tested for interaction reactions of the four milk replacer treatments. All parameter were measured in kg or derivatives thereof.

Phase	Parameter	Treatment (LS Mean$^{Post\text{-}Hoc\ group}$ ± Standard deviation)				Interaction P-value
		SP SC A Biomel	**FP SC B FP-Biomel**	**SP HC C Kalfpap**	**FP HC D FP-Kalfpap**	
TP	Initial weight (Day 0)	39.850^{a} ± 2.664	41.650^{a} ± 3.650	40.025^{a} ± 2.733	41.800^{a} ± 3.909	0.991
	Final weight (Day 77)	96.350^{ab} ± 15.522	64.975^{c} ± 16.346	97.525^{a} ± 8.403	81.300^{b} ± 18.895	0.172
	DMI (MR&SM)	137.036^{a} ± 36.887	91.993^{b} ± 21.141	148.046^{a} ± 13.214	126.646^{a} ± 33.638	0.190
	DMI (MR)	52.138^{a} ± 1.073	52.232^{a} ± 1.109	51.855^{a} ± 0.897	52.395^{a} ± 1.395	0.582
	DMI (SM)	84.898^{a} ± 26.744	39.761^{b} ± 22.003	96.191^{a} ± 13.238	74.251^{a} ± 33.840	0.202
	ADG	0.734^{a} ± 0.201	0.303^{c} ± 0.203	0.747^{a} ± 0.120	0.513^{b} ± 0.214	0.150
	DMI/BW %	0.026^{a} ± 0.003	0.022^{b} ± 0.002	0.028^{a} ± 0.002	0.027^{a} ± 0.004	0.307
	FCR	**2.475^{b} ± 0.250**	**4.961^{a} ± 1.990**	**2.615^{b} ± 0.347**	**3.418^{a} ± 0.714**	**0.036**
P1	Initial weight (Day 0)	39.850^{a} ± 2.664	41.650^{a} ± 3.650	40.025^{a} ± 2.733	41.800^{a} ± 3.909	0.991
	Final weight (Day 63)	78.975^{a} ± 12.524	57.500^{b} ± 11.887	78.875^{a} ± 5.749	68.675^{ab} ± 15.753	0.196
	DMI (MR&SM)	92.663^{ab} ± 17.825	68.382^{c} ± 8.274	100.175^{a} ± 9.407	84.725^{b} ± 17.803	0.382
	DMI (MR)	52.138^{a} ± 1.073	52.232^{a} ± 1.109	51.855^{a} ± 0.897	52.395^{a} ± 1.395	0.582
	DMI (SM)	40.525^{ab} ± 17.596	16.150^{c} ± 8.773	48.320^{a} ± 9.441	32.330^{b} ± 18.085	0.410
	ADG	0.621^{a} ± 0.194	0.252^{b} ± 0.169	0.617^{a} ± 0.093	0.427^{b} ± 0.209	0.151
	DMI/BW %	0.025^{b} ± 0.003	0.022^{b} ± 0.001	0.027^{a} ± 0.002	0.024^{b} ± 0.003	0.931
	FCR	2.460^{c} ± 0.355	5.871^{a} ± 3.252	2.610^{bc} ± 0.312	3.718^{ab} ± 1.409	0.079
P2	Initial weight (Day 63)	78.975^{a} ± 12.524	57.500^{b} ± 11.887	78.875^{a} ± 5.749	68.675^{ab} ± 15.753	0.196
	Final weight (Day 77)	96.350^{ab} ± 15.522	64.975^{c} ± 16.346	97.525^{a} ± 8.403	81.300^{b} ± 18.895	0.172
	DMI (SM)	44.373^{a} ± 9.940	23.611^{b} ± 14.609	47.871^{a} ± 5.477	41.921^{a} ± 16.169	0.099
	ADG	1.241^{ab} ± 0.305	0.534^{c} ± 0.419	1.332^{a} ± 0.273	0.902^{b} ± 0.336	0.256
	DMI/BW %	0.036^{a} ± 0.004	0.026^{b} ± 0.010	0.039^{a} ± 0.004	0.039^{a} ± 0.011	0.079
	FCR	2.589^{a} ± 0.370	* 3.375 ± 2.133	2.633^{a} ± 0.480	3.514^{a} ± 1.564	0.324

^{a,b,c} Means within row with different superscripts differ significantly ($P < 0.05$).

* Due to weight loss by one calf in group B, during P2, FCR was calculated as a negative value, however as less optimal FCR values is represented by larger numbers, a negative value distorts the data disproportionally. Thus, the value was removed, and statistical analysis was done with a missing value (n = 7). It can be presumed that the mean and standard deviation should be larger than the reported values.

Total dry matter intake (DMI) was determined by combining the dry matter intake of the milk replacer (MR) and that of the starter meal (SM), however for more specific insight DMI of milk replacer and starter meal were also calculated separately (Table 3.5). As expected, no significant differences in DMI for the different milk replacers were observed as the volume that was fed was controlled and the same for each treatment group. The DMI differences were thus due to differences in starter meal intake, and it can therefore be assumed that differences in the average starter meal intake were caused by the specific milk replacer. The DMI for starter meal of group B were lower ($P < 0.05$) than all other treatments for all treatment periods, while group C consistently had the highest starter meal intake for all the phases, although not significantly higher than groups A and D (Table 3.5). The lower starter meal DMI and DMI (MR & SM) explains the differences in final body weight.

Average daily gain was influenced by both weight and DMI and therefore followed a similar trend to these parameters (Table 3.5). During P1, the ADG of A and C did not differ, and B and D also did not differ despite both being lower ($P < 0.05$) than A and C. In P2, the same trend continued although B and D differed ($P < 0.05$), and A and D did not differ. For the TP, A and C did not differ, but B and D differed ($P < 0.05$). For all phases B resulted in the poorest ADG (Table 3.5).

Total dry matter intake as percentage of body weight (DMI/BW %) makes it possible to directly compare DMI of the different treatment groups as it takes the size of the calves into account. For both TP and P2, treatments A, C and D did not differ while B were lower ($P < 0.05$) than the other treatment groups. This seems to indicate that due to a low intake, closer to maintenance, the excess energy available was lower, resulting in calves of group B gaining weight less efficiently. It can further be concluded that the higher DMI/BW % were due to a nutrient or nutrients that specifically stimulated intake and the lower values were due to a nutrient or nutrients that reduced appetite. During P1, the weaning phase, DMI/BW% for group C was higher ($P < 0.05$) when compared to all the other treatments and therefore it can be concluded that C stimulated appetite for starter meal.

Since FCR is a function of both ADG and DMI vectors, differences in both would affect FCR and is often used to determine the efficiency of diet use by the animal (Davison *et al.,* 2023). However, FCR alone cannot indicate all the benefits of a milk replacer. A lower FCR value indicates that a lower dry matter unit intake is required to gain 1 kg of body weight (Davison *et al.,* 2023). It should also be noted that DMI of milk replacer and starter meal cannot be separated in FCR calculations. The FCR for TP indicate an interaction ($P = 0.036$), whilst P1 and P2 did not reflect the same interaction (Table 3.5). Although during P2, a calve of group B had lost weight and therefore this value had to be removed from the group when calculating FCR. The FCR for TP can therefore not be considered under main effects and therefore only FCR for P1 and P2 will be considered under main effects. Interaction reaction can be defined as the combination of a diminishing (subtraction) and amplifying (addition) effect (Côté *et al.*, 2016; Fong *et al.*, 2018). In such a reaction only the total or combined effect is quantifiable and therefore the extent of the subtraction or addition effects cannot be determined. Thus, interaction reaction makes it impossible to study the main effects as

there is not a clear indication of the sizes of the main effects. Main effects and multiple parameters can improve understanding of interaction reaction although definite conclusions cannot be reached.

During P1 B and D had the poorest FCR. Treatment D and C did not differ, and the same for A and C (Table 3.5). During P2 all the treatment groups were fed the same starter meal and FCR during this period did not differ amongst the different groups. One of the calves in group B lost weight during this period which might have influenced the results. Although FCR during P2 did not differ, it influenced the FCR for TP where FCR differed. Due to the short period (2 weeks), differences in FCR during P2 may not have manifested as might have been the case if the period was more extended.

The FCR of SC and HC were equal and therefore it could be expected that the weight gain would also be similar as discussed under main effect of carbohydrates (Section 3.4.2). However, the growth performance of the FP group was poorer than that of the group fed SP as discussed under main effects of proteins (Section 3.4.1). These conclusions are in line with the post-hoc groups for the FCR of the TP (Table 3.5) although the p-value indicated an interaction. The TP FCR for treatments A and C is equal and significantly better than B and D. It is suspected that the interaction reaction occurred in D. There are two potential factors that could have led to the interaction reaction. The diminishing effect was due to the presence of trypsin inhibitor in FP (Section 3.4.1.2) and it is hypothesised that the addition effect was due to the presence of HC (Section 3.4.2.2) inducing an increase in starter meal intake.

3.4.1 Protein composition

3.4.1.1 Results

The effect of protein in the milk replacer on growth parameters are shown in Table 3.6.

The initial weight at commencement of the trial were not different between SP and FP. However, at weaning (Day 63) weights were significantly different with SP having a significantly higher weight than FP. On day 77, 2 weeks after weaning, SP was still heavier ($P < 0.05$) and the average difference between the 2 groups increased from 15.837 kg to 23.8 kg indicating that there was a carryover effect after weaning.

Table 3.6 The effect of protein type in the milk replacer on growth performances of calves during the three phases. The protein types are standard protein (SP) and fermented protein (FP). All parameter were measured in kg or derivatives thereof.

Phase	Parameter	Treatments (LS Mean ± Standard deviation)		P-value
		SP	FP	
TP	**Initial weight (Day 0)**	**39.938 ± 2.608**	**41.725 ± 3.654**	**0.135**
	Final weight (Day 77)	96.938 ± 12.073	73.138 ± 19.036	<0.001
	DMI (MR&SM)	142.541 ± 21.241	109.320 ± 32.509	<0.001
	DMI (MR)	**51.997 ± 0.967**	**52.313 ± 1.221**	**0.436**
	DMI (SM)	90.544 ± 21.203	57.006 ± 32.826	<0.001
	ADG	0.740 ± 0.160	0.408 ± 0.229	<0.001
	DMI/BW %	0.027 ± 0.003	0.024 ± 0.004	0.023
	FCR	*2.545 ± 0.301*	*4.189 ± 1.649*	*<0.001*
P1	**Initial weight (Day 0)**	**39.938 ± 2.608**	**41.725 ± 3.654**	**0.135**
	Final weight (Day 63)	78.925 ± 9.414	63.088 ± 14.664	<0.001
	DMI (MR&SM)	96.419 ± 14.304	76.553 ± 15.845	<0.001
	DMI (MR)	**51.997 ± 0.967**	**52.313 ± 1.221**	**0.436**
	DMI (SM)	44.422 ± 14.223	24.240 ± 16.074	<0.001
	ADG	0.619 ± 0.147	0.339 ± 0.204	<0.001
	DMI/BW %	0.026 ± 0.003	0.023 ± 0.002	0.003
	FCR	2.535 ± 0.332	4.795 ± 2.664	0.001
P2	Initial weight (Day 63)	78.925 ± 9.414	63.088 ± 14.664	<0.001
	Final weight (Day 77)	96.938 ± 12.073	73.138 ± 19.036	<0.001
	DMI (SM)	46.122 ± 7.961	32.766 ± 17.635	0.005
	ADG	1.287 ± 0.283	0.718 ± 0.413	<0.001
	DMI/BW %	**0.037 ± 0.004**	**0.033 ± 0.012**	**0.107**
	FCR	**2.611 ± 0.415**	*** 3.449 ± 1.784**	**0.475**

* Due to weight loss by one calf in group B, during P2, FCR was calculated as a negative value, however as less optimal FCR values is represented by larger numbers, a negative value distorts the data disproportionally. Thus, the value was removed, and statistical analysis was done with a missing value (n = 15). It can be presumed that the mean and standard deviation should be larger than the reported values.

The FCR for TP showed an interaction and is not discussed under main effects and is indicated in italics. It is however included in Table 3.6 to ensure completeness. All parameter with P-values larger than 0.05 are indicated in bold for ease of identification.

As the DMI of the milk replacers (MR) was fixed and since the offered volume was consumed in most instances, milk replacer DMI for SP and FP did not differ (Table 3.6). However, for the SP group DMI of starter meal (SM) and therefore the total intake, both before and after weaning, was higher ($P < 0.05$) than for the FP group. This can be attributed to the levels of trypsin inhibitor (Section 3.4.1.2) and supports the findings of Nitsan & Nir (1986).

The SP group showed higher a ADG than the FP group ($P < 0.05$), both before and after weaning. This was expected as ADG is influenced by nutrient intake, and the higher nutrient intake of the SP group can be related to the higher total DMI. Dry matter intake is also positively correlated to body weight (Uskenov *et al.,* 2024) and since the SP group had a higher final body weight, DMI increased. This results in a higher nutrient intake, and this ultimately leads to a higher ADG.

Dry matter intake as percentage of body weight (DMI/BW %) takes the size of the animal into account when comparing DMI. Intake was lower for the FP group compared to the SP group and this resulted in lower body weight. As DMI/BW % compensates for body weight, the smaller size of calves receiving FP were compensated for and intake is directly comparable. During both TP and P1, FP had a lower ($P < 0.05$) DMI/BW % and this was ascribed to a reduction in appetite. The reduction of DMI/BW % was only observed during the milk feeding phase and did not carry over after weaning and this again might be due to the period of P2 being too short to express the differences between the groups.

The FCR for TP showed an interaction, but this was not observed for P1 and P2 (Table 3.5). Regarding main effects, the FCR for SP in P1 was lower ($P < 0.05$) than that of FP, indicating that the SP was better utilized for weight gain than the FP. This finding supports the other parameters and can be attributed to the fact that the anti-nutritional factors in FP might not sufficiently been inhibited for use in milk replacers. In P2 the FCR was similar between SP and FP, which was expected since the starter meal for both treatments were the same and there was no indication that one or the other would more efficiently stimulate rumen development. This data suggest that FP as used in the current trial suppresses appetite in calves. This however confirms that the negative effect of FP does not necessarily carry over after weaning, but it still plays a role as these calves are smaller and do not compensate to fulfil their growth potential.

3.4.1.2 Discussion

The FP was of interest since it has been reported that fermentation can improve digestibility of proteins (Sánchez-García *et al.,* 2024), while also improving the bioavailability of essential amino acids and minerals (Hemalatha *et al.,* 2007; Adeyemo & Onilude, 2013; Çabuk *et al.,* 2018). According to Avilés-Gaxiola *et al.* (2018), fermentation also reduces non-nutritive compounds that inhibit digestive enzymes. It is well documented that the amino acid profile of protein is affected by fermentation and the concentration of some amino acids can increase or decrease (Chaven & Kadam, 1989; Doudu *et al.,* 2003; El-Hag *et al.,* 2002; Pranoto *et al.,* 2013).

To optimise the fermentation of protein, a separate trial was conducted on the raw materials before it was used in the milk replacers. A proximate, fatty acid and amino acid analyses were performed on the fermented plant protein (Cordy, 2024; Table 3.1). The optimum fermentation temperature and fermentation time was established at 40°C and 7 days respectively (Cordy, 2024). During the

optimalisation of the fermentation process a standardised process was established for commercial production by Nandrea Health Products. To assess the value of FP, it was compared with other high quality, common protein sources and this is shown in Table 3.7. It should be noted that the values of the other protein sources were obtained from literature and this is therefore not ideal for direct comparison.

Table 3.7 Amino acid analysis of fermented protein (FP) recalculated to a comparable total in comparison to soybean meal and egg albumin. All values converted to g/100g protein.

Amino acid		**Full fat soya** (Gao *et al.*, 2013)	**FP** (Recalculated from Cordy, 2024)	**Egg albumin** (Dajnowska *et al.,* 2023)
Histidine	His	2.4	2.8	2.3
Arginine	Arg	6.5	6.9	5.6
Serine	Ser	4.9	4.9	7.2
Glycine	Gly	3.8	4.4	3.5
Aspartic acid	Asp	10.5	10.9	
Glutamic acid	Glu	19.3	16.4	13.7
Threonine	Thr	3.7	4.1	4.6
Alanine	Ala	3.8	4.1	5.9
Proline	Pro	4.0	5.2	3.3
Lysine	Lys	6.1	5.1	7.0
Tyrosine	Tyr	5.6	4.1	3.7
Methionine	Met	1.3	2.0	4.7
Valine	Val	0.5	4.9	6.6
Isoleucine	Ile	4.0	4.6	5.4
Leucine	Leu	7.3	7.8	8.7
Phenylalanine	Phe	3.1	5.6	5.9
Cysteine	Cys	7.9		3.8
Total		94.7	94.4	91.9

In support to the findings of Frais *et al.* (2008) and Teng *et al.* (2012), the fermentation of soybean meal improved the composition of the amino acid ratio, but due to the insufficient inhibition of trypsin inhibitor the inclusion of FP in the milk replacer did not translate in improved calf performances (Table 3.6). Although it was clear that fermentation had indeed been successful further analysis were performed to explain the contradiction to expectations.

Untreated plant meal such as soybean meal, contain antinutritional factors (e.g., trypsin inhibitor) that can affect protein digestibility and the availability of amino acids (Jahan-Mihan *et al.,* 2011). Feed processing methods such as heating, re-roasting, and extrusion have been shown to successfully inhibit these antinutritional factors (Akande & Fabiyi, 2010). However, overheating makes amino acids unavailable due to a complex reaction with sugars including browning or the Maillard reaction (El-shemy *et al.,* 2000).

As lactic acid bacteria were used in the fermentation process, it was expected that this bacterial fermentation would denature the trypsin inhibitor (Gao *et al.,* 2013; Emkani *et al.,* 2022). The FP was also pasteurised before spray-dried during the production process to reduce the *Lactobacillus* concentration and mitigate the risk of pathogenic organisms (Nandrea Health Products). The pasteurization temperature was however possibly not high enough to degrade the antinutritional factors.

Lalles *et al.* (1996) reported that a trypsin inhibitor content of below 4 mg/g protein resulted in an apparent digestibility of more than 80% of soy protein and it was expected that fermentation and spray-drying would ensure a similar digestibility. As can be seen in Table 3.8 this was not achieved, and the poor results can be attributed to a trypsin inhibitor level of 9.2 mg/g protein.

Table 3.8 Trypsin inhibitor and Beta-conglycinin values of FP as analysed by Hamlet Protein, Horsens, Denmark.

Analysis name	Result	Unit	Method
Protein (as is)	36.99	%	Dumas
DM	91.04	%	103°C 4 hours/EU 152/2009
Protein DM	40.6	%	
Trypsin Inhibitor TIA (TIU/1900) mg/sample	9.2	mg/g sample	
Trypsin inhibitor TIA (TIU/1900/protein as is) mg/g protein	25.0	mg/g protein	
Beta-conglycinin	1.2	ppm	ELISA
Stachyose	0.1	%	TLC
Raffinose	0.6	%	TLC
Galactose	4.0	%	TLC
pH in 10% suspension	4.5		

The two main seed storage proteins in soybean are glycinin and β-conglycinin (Nielsen,1985) with a reported content of 32% and 23%, respectively (Iwabuchi & Yamauchi 1987). In calves, levels of 14 and 12 mg/g of glycinin and β-conglycinin reduces the duration of the jejunal migrating motor complexes causing a decrease in the mean duration of phase I (quiescence) (Lalles *et al.,* 1995). It was therefore anticipated that the fermentation process of the soymeal, yielding 1.2 ppm (µg/g) β-conglycinin (Table 3.8), therefore reduced this anti-nutrient to levels that allows the safe inclusion of FP in calf milk replacers.

Trypsin inhibitor strongly inhibit the activity of key pancreatic enzymes trypsin and chymotrypsin thereby reducing digestion and absorption of dietary protein by the formation of complexes that are indigestible even in the presence of high amounts of digestive enzymes (Gemede & Ratta, 2014; Avilés-Gaxiola *et al.,* 2018). There are multiple ways to process soya although not all methods are conducive to optimal result for milk replacers of pre-ruminants. The first way trypsin inhibition levels could be addressed is through physical processing, although this might not always be optimal under

these circumstances. Avilés-Gaxiola *et al.* (2018) compiled a summary of research on trypsin inhibitor inactivation methods (Figure 3.2).

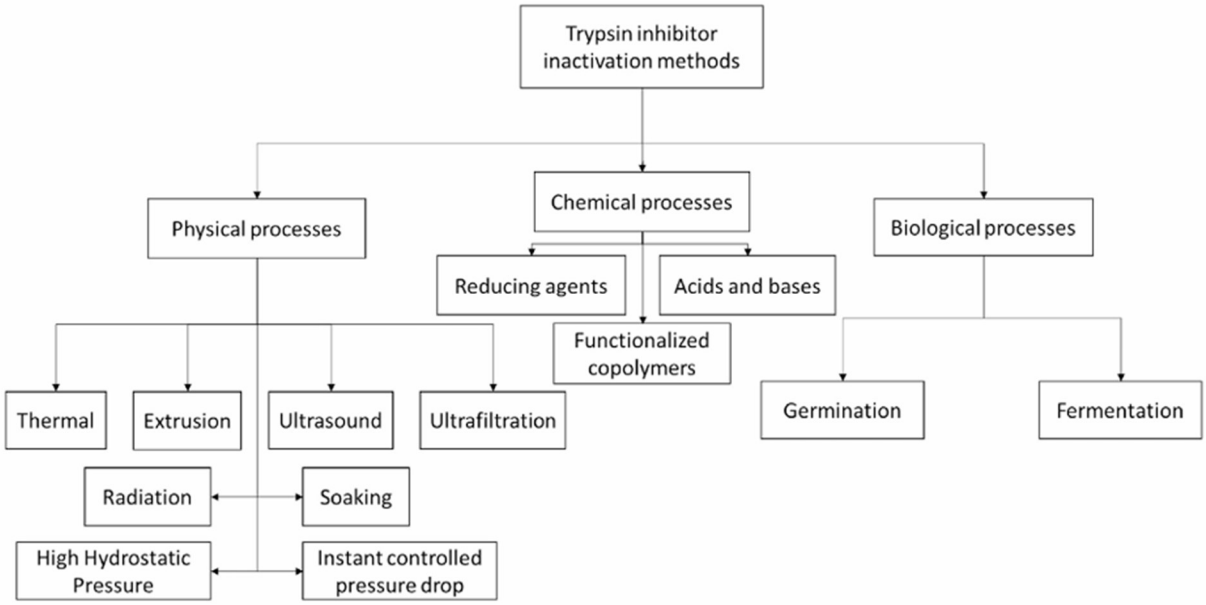

Figure 3.2 Classification of the methods used to inactivate trypsin inhibitor (Avilés-Gaxiola *et al.,* 2018).

As soybean oilcake meal is a by-product from the extraction of soy oil, this is a relatively low-cost product with a high protein content. It has a well-balanced amino acid profile and constant composition (Feedipedia, 2024). Due to large scale production ensuring a steady supply, relative to other traditional protein sources, these factors contribute to making it a preferred raw material in animal nutrition (Storebakken *et al.,* 2000). Thus, soybean meal is increasingly acceptable as the protein source in animal feed even with the limitation of use due to the content of anti-nutritional factors (Francis *et al*., 2001). Trypsin inhibitors is one of the main anti-nutritional factors in soya and this inhibits the digestive enzyme trypsin with very high specificity. This impairs the digestive functions in the gut of animals and has been the reason for numerous studies on the processing of soybean meal to reduce the impact of anti-nutritional factors (Hoffmann *et al.,* 2003).

Fermentation is a processing method often used to degrade trypsin inhibitor (Figure 3.2), but results of the current study has shown that, with the specific organism used, the levels of trypsin inhibitor did not decrease to the extent where it could be included in pre-ruminant milk replacers without affecting the calves. To effectively use FP in future calf milk replacers, the inhibition of trypsin inhibitor will have to be addressed. As the production process has already been established at Nandrea Health Products in Oudtshoorn, it would be more conducive to amend the process rather than establishing another raw material or processing method. The least invasive intervention would be to alter the inoculum used for fermentation rather than adding a specific physical processing method as suggested by Avilés-Gaxiola *et al.* (2018).

Gao *et al.* (2013) conducted an in-depth study on *Lactobacillus brevis* and *Aspergillus oryzae* determining the optimal fermentation process as well as complete analysis on the changes of amino acids, phytic acid, crude protein, crude fat, crude fibre and urease activity when fermenting soybean meal. This study reported a 57.1% reduction in trypsin inhibitors when fermenting with *Lactobacillus brevis* and 89.2% when fermenting with *Aspergillus oryzae* and a 6.4% increase in CP content for *Lactobacillus brevis* and 12.9% for *Aspergillus oryzae*.

Other than adding the organisms to the fermentation, the preparation of the organisms for the inoculum should be considered. With the already established process at Nandrea Health Products, the preparation of an inoculum through anaerobic incubation of *Lactobacillus brevis* with MRS agar at 37°C for 3 days is similar as for the other *Lactobacillus* species already used in the inoculum. The facilities at Nandrea Health Products laboratory can accommodate *Aspergillus oryzae* transfer to Potato-Dextrose Agar and incubation at 28°C for 5 days as many of the quality control procedures follow a similar established process (Gao *et al.,* 2013; Mora-Lugo *et al.*, 2014).

Lactic acid bacteria are a heterogeneous group of bacteria that are generally regarded as safe (Plavec & Berlec, 2020). Adeyemo & Onilude (2013) isolated nine strains of *Lactobacillus plantarum* from a spontaneous fermentation and these were selected in terms of their alpha-galactosidase production and used for the fermentation of legumes. Over a 5-day fermentation period the reduction of anti-nutritional factors was monitored at 24-hour intervals. Trypsin inhibitor was found to be reduced from 1.20 mg/g to 0.010 mg/g and the production of alpha-galactosidase by *Lactobacillus plantarum* (1.8 unit/ml) enhanced the reduction. When added to a soyabean food blend, this organism improved the nutritional composition significantly.

Zhang *et al.* (2022) reported that *Lactobacillus paracasei* fermentation significantly decreased the level of anti-nutritional factors in soybean meal since it was shown to lower glycinin by 44.05%, β-conglycinin by 31.10%, and trypsin inhibitors by 76.57%.

In conclusion, although the evaluation of the bioavailability and improvement of the amino acid composition of FP through a growth trial was the aim of the current trial, it was concluded that the current FP produced is not suitable to be used in pre-ruminants since the levels of trypsin-inhibitor was higher than anticipated. This resulted in sub optimal growth for calves on milk replacer where FP was included as a raw material.

Therefore, milk replacers containing FP performed poorly due to high levels of trypsin inhibitor, therefore unless FP is treated to reduce the trypsin inhibitor levels to lower than 4 mg/g protein (Lalles *et al*., 1996), it should not be included in milk replacer for neo-natal calves. These results support the findings reported by Nisley *et al.* (2024), but contrasts with the findings of Kakade *et al.* (1976) and Ansia & Drackley (2020). Although it should be noted that these calves were weaned, and pepsin hydrolysis takes place during fermentation in the rumen (Mir *et al.*, 1989), inactivating antigenic capacity of anti-nutrients present in soya (Barratt *et al.*, 1978).

3.4.2 Carbohydrate composition

3.4.2.1 Results

In this Section the main effect, standard level of carbohydrates (SC) is compared to the higher level of carbohydrate (HC) in milk replacers. The effect of carbohydrate in the milk replacer on growth parameters are shown in Table 3.9. The FCR for TP showed an interaction and can thus not be discussed under main effects and are indicated in italics. For ease of identification all parameter with P-values < 0.05 were presented in bold.

The initial weight of the animals at commencement of the trial were not different between SC and HC groups, therefore neither treatment started with an advantage. No weight differences were observed on day 63 nor 77 indicating that SC and HC did not influence body weight for either of these 2 periods. As the aim with P2 was to determine the effect of wean shock, it was only a two-week period, and this may not have been a sufficiently long period to reveal differences between the 2 groups.

Table 3.9 The effect of carbohydrate inclusion level in the milk replacer on growth performances of calves during the three phases. Treatments are standard carbohydrate (SC) inclusion and higher carbohydrate (HC) inclusion. All parameters were measured in kg or derivatives thereof.

Phase	Parameter	Treatments		P-value
		SC	HC	
TP	Initial weight	40.750 ± 3.224	40.913 ± 3.385	0.890
	Final weight	80.663 ± 22.353	89.413 ± 16.424	0.117
	DMI (MR&SM)	**114.514 ± 32.969**	**137.346 ± 27.049**	**0.015**
	DMI (MR)	52.185 ± 1.056	52.125 ± 1.167	0.882
	DMI (SM)	**62.329 ± 33.211**	**85.221 ± 27.287**	**0.015**
	ADG	0.518 ± 0.296	0.630 ± 0.207	0.105
	DMI/BW %	**0.024 ± 0.003**	**0.027 ± 0.003**	**0.007**
	FCR	*3.718 ± 1.877*	*3.016 ± 0.682*	*0.076*
P1	Initial weight	40.750 ± 3.224	40.913 ± 3.385	0.890
	Final weight	68.238 ± 16.190	73.775 ± 12.609	0.204
	DMI (MR&SM)	**80.523 ± 18.370**	**92.450 ± 15.901**	**0.023**
	DMI (MR)	52.185 ± 1.056	52.125 ± 1.167	0.882
	DMI (SM)	**28.338 ± 18.408**	**40.325 ± 16.199**	**0.024**
	ADG	0.436 ± 0.259	0.522 ± 0.184	0.171
	DMI/BW %	**0.023 ± 0.003**	**0.026 ± 0.003**	**0.009**
	FCR	4.165 ± 2.846	3.164 ± 1.140	0.124
P2	Initial weight	68.238 ± 16.190	73.775 ± 12.609	0.204
	Final weight	80.663 ± 22.352	89.413 ± 16.424	0.117
	DMI (SM)	**33.992 ± 16.145**	**44.896 ± 12.060**	**0.018**
	ADG	0.888 ± 0.509	1.117 ± 0.370	0.065
	DMI/BW %	**0.031 ± 0.009**	**0.039 ± 0.008**	**0.008**
	FCR	* 2.956 ± 1.477	3.073 ± 1.207	0.317

* Due to weight loss by one calf in group B, during P2, FCR was calculated as a negative value, however as less optimal FCR values is represented by larger numbers, a negative value distorts the data disproportionally. Thus, the value was removed, and statistical analysis was done with a missing value (n = 15). It can be presumed that the mean and standard deviation should be larger than the reported values.

As expected, dry matter intake for SP and FP did not differ, since milk replacer (MR) intake was controlled (Table 3.9). Starter meal (SM) intake for each of the phases was higher ($P < 0.05$) for HC than SC. This suggest that HC stimulates appetite. The increased starter meal intake stimulates rumen development (Diao *et al*., 2019), leading to a further increase in intake of starter meal and this continued for the observed trial period after weaning.

Although the average ADG of the HC group was numerically higher during each of the three respective phases, it was not significant (Table 3.9). This can be attributed to the fact that during P1 the main source of energy was the milk replacer and during P2 the period was perhaps too short to show any meaningful differences.

The DMI/BW % for HC were higher ($P < 0.05$) than for SC for all the phases (Table 3.9). This can be attributed to the higher ($P < 0.05$) starter meal intake of the HC group. As feed conversion efficiency is improved by milk replacers which are enzymatically digested compared with starter meal which is degraded by microorganisms in the rumen (Nejad *et al*., 2013), the higher starter meal intake would result in a less favourable DMI/BW %.

The FCR in TP showed an interaction but this was not the case for P1 and P2 (Table 3.5), and they are therefore discussed under main effect of carbohydrates. Since HC stimulates intake of starter meal, it was expected that this would lead to improved efficiency, especially during P2. However, FCR did not differ between the HC and SC groups either before or after weaning (Table 3.9). Due to the higher ($P < 0.05$) dry matter intake of calves on HC, improved FCR was expected compared to calves fed SC since the higher intake of HC should have allowed for a bigger differential between maintenance and energy available for growth. It was further expected that this would be enhanced during P2 because of the improved rumen development. Although it should be considered that P2 was only a 2-week period and in shorter periods FCR determination is less optimal as slight fluctuation can have larger impacts. Further, the effect of increased dry matter intake should be considered as an increase in dry matter intake leads to an elevation in the metabolism (Van Hoeij *et al.*, 2019) and any increase in dry matter intake also results in a decrease in feed utilization (Colucci *et al.*, 1982).

3.4.2.2 Discussion

As highlighted in Section 3.2, pre-ruminants can readily utilize glucose, galactose and lactose, but has a limited ability to utilize maltose and starches and is unable to utilise sucrose (Dollar & Porter, 1957; Walker, 1959; Estévez *et al.,* 2014). However, maltose activity gradually increases and in two-week-old calves the enzyme activity reaches half the level of activity of adult cattle (Krehbiel *et al.,* 1996; Le Huerou *et al.,* 1992). This suggests that high starch content in milk replacers could stimulate dry matter intake of starter meal, as supported by the data of the current study (Table 3.9), leading to an increase in rumen development. Based on the performance of the calves, it would appear as if maltose activity can be stimulated by the consumption and therefore exposure to starch. The stimulated enzyme activity, of enzymes such as maltose and isomaltose, will lead to improved digestion in the abomasum which will indirectly stimulate the development of the rumen (Diao *et al*., 2019). During P1 the calves relied to a large degree on enzymatic digestion in the abomasum, but over time the importance of ruminal digestion through fermentation increased.

The HC was investigated for its positive effect on wean shock and thus its ability to stimulate starter meal intake during the liquid (milk) phase of the calf's life. This effect carries over after weaning as it accelerates rumen development. Although HC did stimulate appetite and thus starter meal intake, FCR did not improve as starter meal is broken down in the rumen and not digested enzymatically and this is a less efficient process compared to enzymatic digestion (Ganai *et al.*, 2019). As there

was an increase in starter meal intake after weaning no retardation in growth was observed and thus no wean shock although calves on the SC started from a lower basis (Table 3.9).

These results confirmed that milk replacer high in carbohydrates can be used and that growth performance is equal to that of conventional milk replacer. This finding is in contrast with that of Huber *et al.* (1968). This can be explained by the fact that calves in their reported trials were started on high carbohydrate milk replacer between 3 and 4 days after birth. At that stage the digestion of carbohydrates is inefficient as the secretion of enzymes such as isomaltose and maltose are inadequate (Le Huerou *et al.,* 1992). In this trial the transition to a high carbohydrate milk replacer started only on day 11 and this was in line with the findings of Krehbiel *et al.* (1996) who reported that maltase activity at that age reached half of that of adult cattle. Bernard *et al.* (2013) reported that the inclusion of 10 to 20% corn syrup solids and dextrose has no negative effect on health and performance of newborn dairy calves from birth through weaning. This is in line with our finding although they had no transition phase and started feeding milk replacers high in carbohydrates after the calves had received colostrum and transition milk for 2 days. As corn syrup is composed of glucose molecules and not complex carbohydrates this might explain why adequate utilization occurred at an earlier age.

3.5 Conclusion

Milk replacers containing FP performed poorly due to high levels of trypsin inhibitor, therefore unless FP is treated to reduce the trypsin inhibitor levels to lower than 4 mg/g protein, it should not be included in milk replacer for neo-natal calves.

This study confirmed that milk replacer high in carbohydrates can be used and that growth performance is equal to that of conventional milk replacer.

It was concluded that the feeding of high carbohydrate milk replacers had an additional benefit in that it stimulated starter meal intake. No other studies reporting this finding could be found in the literature and this appears to be novel, and further studies should be conducted to verify or disprove this.

3.6 References

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Chapter 4
The effect of using different protein sources and high level of carbohydrates in milk replacers on the profitability of rearing Holstein bull calves

4.1 Abstract

The economic viability of four milk replacers was evaluated to determine the most cost-effective option for rearing Holstein bull calves. Thirty-two calves were randomly assigned to one of four treatments (A, B, C, D) in a 77-day trial, divided into two phases: Phase 1 (P1, days 0–63) during which the milk replacers were fed, and Phase 2 (P2, days 63–77), a two-week post-weaning period. Milk replacer intake was controlled, while starter meal was offered *ad libitum*, with all intakes and refusals recorded.

Several financial parameters were calculated, including total cost, average daily cost (ADC), cost per weight gain (Cost/WD), and income from the sale of calves at the end of the trial. Cost per weight difference during P2 was the only parameter that did not differ between treatments ($P > 0.05$), with all other parameters differing between treatments.

In terms of cost and ADC, treatment D was the most economical with an average feeding cost for the trial (TP) at R2739.40/calf. However, when factoring in feed conversion ratio (FCR), treatment C emerged as the most cost-effective, with a total feeding cost of R3317.70/calf and an ADC of R43.09/calf. For the entire trial period (TP), treatment C also performed best in terms of income and Cost/WD, despite not differing significantly from treatments A and D. Treatment C had the lowest average Cost/WD at R58.83 and the highest ($P < 0.05$) income compared to treatment B, albeit not different from Treatments A and D. Based on these results, treatment C is recommended as the most cost-effective milk replacer.

4.2 Introduction

The milk replacer industry contributes to animal production and plays a significant role in the animal husbandry sector (Göncü *et al*., 2023). Milk replacers refer to products that substitute or supplement mother's milk during the suckling phase of mammalian animals (Soberon *et al*., 2012). As these milk replacers are commonly used to replace natural milk, it is formulated to mimic the nutrient value of natural milk. Commercial milk replacers must therefore provide adequate nutrients to ensure the health and growth of suckling neonatal animals (Palczynski *et al*., 2020).

There are various reasons and situations in which milk replacers are used and the growing demand has led to the rapid expansion of the milk replacer industry (Kertz *et al*., 2017). The use of milk

replacers ensures a consistent supply of nutrients to young animals under circumstances where the dam's milk is unavailable. This commonly happens in the commercial dairy industry where replacement heifers are reared on milk replacer and the milk produced by the dam is sold. Milk replacers are also used to mitigate the risk of disease transmission from the dam to the offspring (Nielsen *et al.*, 2008; Van Niekerk *et al*., 2021). Milk replacers are formulated to provide optimal growth, increase productivity and can provide a more flexible management option to farmers. According to Akins (2016), milk replacers can supplement mother's milk and partially replace it, providing farmers with a way to control the cost of rearing animals. As the milk composition of different species vary significantly (Gantner, 2015), milk replacers are formulated to be specie specific, providing research and development opportunities.

Compared to the global market, the South African milk replacer market is relatively small. In monetary terms, the South African milk replacer market was estimated at between R30 and R35 billion per annum (Grobler, 2008). However, due to constantly changing economic environment and the lack of data, the value may be significantly different.

One of the biggest challenges for this sector is the high production and retail cost of milk replacers (Carulla *et al.,* 2023). Although this problem is not exclusive to the South African milk replacer industry, its impact is significant, affecting both small scale farmers (Syomiti *et al.,* 2014) and large commercial producers (Sharpe & Heins, 2021). The high cost of milk replacers can be directly attributed to the expensive, high quality raw materials needed to produce it (McCoard *et al*., 2021). This high cost often forces farmers to consider less well-balanced alternatives such as whey sweepings and rejected baby milk, which can negatively impact growth rates and overall productivity (Fischer *et al*., 2019; Creutzinger *et al*., 2021). Since a significant amount of milk replacers used in Southern Africa is developed and manufactured in first world countries the unfavourable exchange rate further exacerbates cost challenges for using imported milk replacers in the local market (Aron *et al.,* 2014).

The fact that different milk replacers influence growth rate and the incidence of diarrhoea in calves is well documented (Blome *et al.*, 2003; Amado *et al.*, 2019; Li *et al.*, 2019). This effect differs depending on the specific rearing conditions. The use of different protein sources and different energy sources impact on the performance of calves and the cost of the milk replacer significantly impacts rearing cost. The biological and economic viability of a specific feeding regime depends on growth rate, feed conversion, cost of the milk replacer and the incidence of growth insults caused by factors such as diarrhoea (Wei *et al*., 2023).

To provide more affordable options to farmers, alternative sources of affordable raw materials are required (Bezuidenhout *et al.*, 2019). The other challenge that cannot be separated from the cost of the milk replacer is the quality of the milk replacer to provide optimal growth and development for the production animals (Le Roux *et al.*, 2019). Therefore, Kertz *et al*. (2011) propose that research should be conducted on more affordable and higher quality milk replacers.

In this study a higher carbohydrate level and an alternative protein source in milk replacers was investigated with the goal to find more affordable alternative sources of these raw materials to be able to provide cheaper milk replacers without compromising the quality of the final product. During digestion, starch is broken down to glucose and absorbed into the bloodstream (Lee *et al.*, 2012). Glucose functions either as an energy source or is stored by the liver as glycogen for later use (Van Soest, 1994). Maltase and isomaltase digest starch to glucose and this can serve as the source of glucose in milk replacers. The pre-cooked maize in high carbohydrate milk replacers (Chapter 3) can provide a cost saving effect as the cost of high carbohydrate milk replacers is significantly lower compared to conventional milk replacers. It was therefore hypothesized that if the growth rate and incidence of diarrhoea of calves fed the high carbohydrate milk replacer is equal or better than that of calves fed a standard commercial milk replacer, this will imply a decrease in the cost of rearing calves.

Fermented plant protein developed by Nandrea Health Products contain a high fat content (20%) and this serves as part of the fat required in milk replacers. As fat is an expensive feed fraction in milk replacers, this could possibly reduce the cost of a milk replacer. Although the fermentation of the plant protein is aimed at improving the amino acid composition in fermented protein, the high fat content is an added advantage and ensures a cost saving when compared to the standard protein sources used in commercial milk replacers. If the performance of calves fed milk replacers containing fermented protein is equal or better than that of calves fed a standard commercial milk replacer, it could be advantageous to use fermented protein when formulating milk replacers. If the growth performance of these calves is equal to that of calves fed the standard milk replacer, this will also have a cost saving effect. Therefore, a study was conducted at Livestock Wellness, George to evaluate the effect of fermented protein and high carbohydrate milk replacers on profitability parameters when used to rear neonatal dairy calves.

4.3 Materials and methods

Ethical clearance for this study was obtained from the UFS Animal Research Ethics Committee for all trial related practices (UFS-AED2023/0051).

The biological effect of higher carbohydrate levels and fermented protein has been discussed in detail in Chapter 3. In the current chapter the focus is on the financial aspects and the cost benefit analysis will be discussed. Although the trial design and milk replacer production processes were discussed in Chapter 3, Section 3.3, it is briefly summarised here as it is also relevant when economic factors are discussed.

The fermented protein was produced by Nandrea Health Products (Chapter 3, Table 3.1), and the calf rearing trial took place at Livestock Wellness in George. Table 4.1 shows the experimental design of the trial, and the four treatment groups used.

Table 4.1 The experimental design of the trial, with a total of 32 calves (N = 32) allocated to four different treatments (n = 8). As the cost and efficacy of four different milk replacers are compared in this chapter as opposed to the different raw material compared in Chapter 3 the four treatments are designated as A, B, C and D.

	Standard protein (SP)	**Fermented protein (FP)**
Standard carbohydrate (SC) inclusion levels	n = 8 (Biomel®) **A**	n = 8 (FP-Biomel) **B**
Higher carbohydrate (HC) inclusion levels	n = 8 (Kalfpap®) **C**	n = 8 (FP-Kalfpap) **D**

During the trial a regime of 3 feedings of 2 L milk replacer per day was used. On day 1 of the trial, the calves received 1 L per feeding, and this was increased in steps of 200 ml per feeding per day until 2 L per feeding was reached. At the end of the trial the milk replacers were gradually decreased to 1 L and 2 feedings per day (See Appendix A, Table A.1). After weaning, the calves were kept in the unit for a further 2 weeks. During this period, they were fed starter meal *ad libitum.* Any milk replacer refusals were noted, and total milk intake was compounded on a weekly basis. As discussed in Chapter 3, Section 3.3, random samples of milk replacers and the starter meal was collected for proximate analysis (Chapter 3, Table 3.4). Milk replacers were formulated on an iso-caloric and iso-nitrogenous basis. The protein content was determined according to standard methods prescribed by AOAC (2002) using the Dumas method and a LECO FP828 (St Joseph, MI, USA). The average metabolic energy of the milk replacers was 12.44 MJ/kg and the average nitrogen content was 32.2 g/kg with a crude protein content of 201.25 g/kg (Table 3.4). The crude fat content of the milk replacers was gravimetrically determined by Assurecloud, (Evers *et al.*, 2000), as proximate analysis (ether extract) does not efficiently extract emulsified fats (Moneeb *et al.*, 2021). Milk replacers had an average crude fat of 157.3 g/kg for milk replacers with standard carbohydrate inclusion levels and 115.85 g/kg for milk replacer with high carbohydrate inclusion levels as the carbohydrate compensated in terms of energy for the lower fat content (Table 3.4). During the calf rearing trial, a standard commercial starter meal with a metabolizable energy content of 10.57 MJ/kg, crude protein and crude fat content of 154.4 g/kg and 3.2 g/kg respectively, was used (Chapter 3, Table 3.4). Crude fat content adhered to general guidelines for ruminating cattle (Fiorentini *et al.*, 2015; Bionaz *et al.*, 2020).

Thirty-two Holstein bull calves, supplied by a commercial supplier, were marked with different numbered colour tags and randomly allocated to the respective treatment groups. All the calves received colostrum directly after birth and entered the trial at an age of between 3 and 4 days. Calves fed the high carbohydrate milk replacers were gradually transitioned from standard milk replacer to the high carbohydrate milk replacers. This was done to limit the incidence of diarrhoea due to a change in the diet. It is well documented that when calves are 11 days old the activity levels of starch digestive enzymes including amylase, maltase and isomaltase should have increased to such an extent that it is possible to successfully introduce high carbohydrate milk replacers (Miyashige &

Yahat, 1980; Harmon, 1993). Harmon (1993) further concludes that the secretion and activity of amylase activity can be manipulated nutritionally. During the current trial this transition started with the inclusion of 25% carbohydrate milk replacer on day 11 and was increased in fractions of 25%, reaching 100% on day 14. As the cost of high carbohydrate milk replacers is less than conventional milk replacers this transition is important as it has a direct bearing on the total cost of weaning a calf (Table 4.2).

A standard vaccination program was followed (Appendix A), and calves were weighed on a weekly basis. Table 4.2 shows the prices per kg milk replacer (February 2024) as were provided by Nandrea Health products, as well as the prices per kg of the starter meal. The sale price of calves used in calculations was obtained from the Red Meat Producers Organisation (RPO) price report on 10/03/2024.

Table 4.2 The prices used when calculating the parameter used in the cost benefit analysis (Nandrea Health Products; RPO).

Item	**R/kg**
Biomel (A)	58.25
FP-Biomel (B)	50.47
Kalfpap (C)	47.95
FP-Kalfpap (D)	39.20
Starter meal	7.53
Live weight	36.11

The financial viability of the different treatments was evaluated using a cost benefit analysis. For analysis of the financial data a one-way ANOVA was used as the different milk replacers were compared and not individual nutrients. Table 4.3 shows the hypotheses for both the nutrient analysis as well as the financial analyses to provide a better understanding as to why the analysis for the chapter differs. Significance was declared at differences of $P \leq 0.05$ and tendencies at $P \leq 0.10$.

Table 4.3 The null hypothesis, alternative hypothesis and interpretation of P-values for both research chapters.

		Null and alternative hypotheses	P-value interpretation	
Chapter 3	**Interaction**	H_0: An interaction is absent H_A: An interaction is present	p-value ≤ 0.05 {Reject H_0	Interaction
			p-value > 0.05 {Accept H_0	No interaction
	Protein main effect	H_0: $\mu_{SP} = \mu_{FP}$ H_A: $\mu_{SP} \neq \mu_{FP}$	p-value ≤ 0.05 {Reject H_0	$\mu_{SP} \neq \mu_{FP}$
			p-value > 0.05 {Accept H_0	$\mu_{SP} = \mu_{FP}$
	Carbohydrate main effect	H_0: $\mu_{SC} = \mu_{HC}$ H_A: $\mu_{SC} \neq \mu_{HC}$	p-value ≤ 0.05 {Reject H_0	$\mu_{SC} \neq \mu_{HC}$
			p-value > 0.05 {Accept H_0	$\mu_{SC} = \mu_{HC}$
Chapter 4	**One-way ANOVA**	H_0: $\mu_A = \mu_B = \mu_C = \mu_D$ H_A: At least one group mean is significantly different	p-value ≤ 0.05 {Reject H_0	Further investigation with Post-Hoc test
			p-value > 0.05 {Accept H_0	$\mu_A = \mu_B = \mu_C = \mu_D$

Figure 4.1 depicts the phases used to analyse the financial data and these were used throughout the trial. Phase 1 (P1) refers to the period during which the calves were fed milk replacer and was from day 0 – 63. Day 63 was also the day on which the calves were weaned. Phase 2 (P2) was the two-week observation period after weaning, starting on day 64 and terminating on day 77. During this phase the calves had *ad libitum* access to starter meal as the only food source. The total Phase (TP) covered the period from day 0 to 77 and included both P1 and P2.

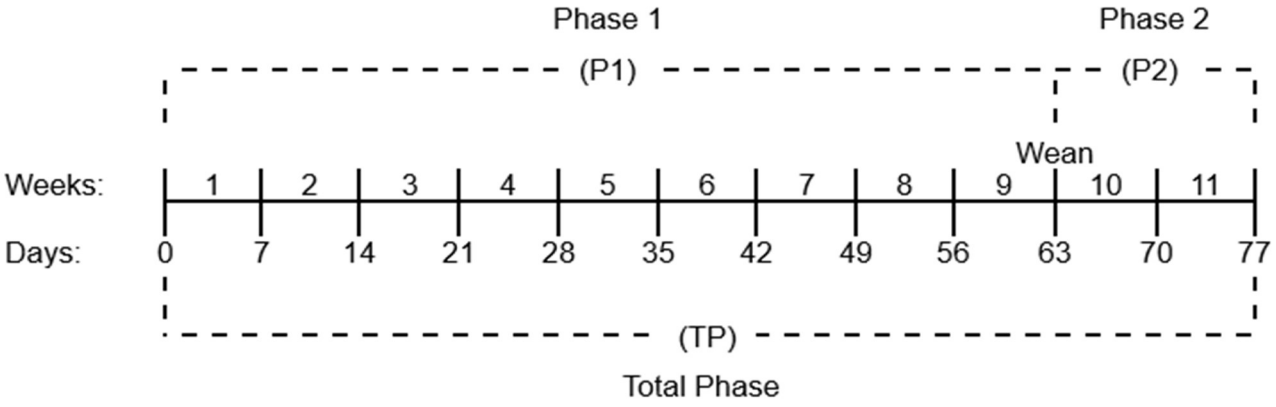

Figure 4.1 A line diagram of the time periods used in the trial.

4.4 Results and Discussion

Although financial viability of the different treatments evaluates milk replacers and not individual nutrients, growth performance cannot be completely separated from cost benefit. Thus, a summary of the growth performance parameters investigated in Chapter 3 is provided. Milk replacers containing FP performed poorly due to high levels of trypsin inhibitor, therefore unless FP is treated to reduce the trypsin inhibitor levels to lower than 4 mg/g protein (Lalles *et al*., 1996), it should not be included in milk replacers for neo-natal calves.

Data from Chapter 3 confirmed that a milk replacer high in carbohydrates can be used and that growth performance is equal to that of a conventional milk replacer. In this trial the transition to a high carbohydrate milk replacer only started on day 11 to comply with the findings of Krehbiel *et al.* (1996) who reported that maltase activity at that age reached half of that of adult cattle. It was concluded that the feeding of high carbohydrate milk replacers had an additional benefit in that it stimulated starter meal intake. No other studies reporting this finding could be found in literature and this appears to be novel. It was lastly concluded in Chapter 3 that further studies should be conducted to verify or disprove this finding.

In Chapter 3 the protein type (SC or FP) and carbohydrate inclusion levels (SC or HC) were investigated. In this chapter the emphasis is on the different milk replacers and the aim of the investigation was to evaluate the economic viability of the different types of milk replacers. The protein type and carbohydrate inclusion levels do however have a significant impact on the cost and therefore the economic viability of these milk replacers. Therefore, growth performance parameters in Chapter 3 (Table 3.5) also need to be considered when discussing specific profitability parameters.

In Table 4.4 the effect of the different milk replacers on profitability parameters of calves over all phases are shown. The parameters reported includes cost, average daily cost (ADC), cost per weight difference (Cost/WD) and income, based on the economic value of the calve at weaning and at the end of the trial.

Cost per weight difference during P2 (displayed in bold in Table 4.4) was the only parameter that did not differ between treatments with all other parameters differing among treatments. Cost per weight difference during P2 of treatment B (FP-Kalfpap) are represented by 7 calves only resulting in higher variation which most likely affected the significance. All other cost benefit parameters analysed in Table 4.4 had $P < 0.05$ leading to the rejection of the null hypotheses. This implies that groups in rows were not significantly equal.

Table 4.4 The effect (mean ± SE) of milk replacer on profitability parameters over all phases of calves expressed in terms of ZAR.

Phase	Parameter	Treatment (LS Mean$^{Post\text{-}Hoc\ group}$ ± Standard deviation)				P-value
		SP SC A Biomel	**FP SC B FP-Biomel**	**SP HC C Kalfpap**	**FP HC D FP-Kalfpap**	
TP	Cost (MR&SM)	3676.33^{a} ± 217.54	2935.54^{c} ± 126.46	3317.70^{b} ± 107.90	2739.40^{d} ± 249.52	<0.001
	Cost (MR)	3037.05^{a} ± 62.53	2636.14^{b} ± 55.97	2593.38^{b} ± 48.94	2180.29^{c} ± 62.00	<0.001
	Cost (SM)	639.28^{a} ± 201.38	299.40^{b} ± 165.68	724.32^{a} ± 99.68	559.11^{a} ± 254.82	<0.001
	ADC	47.75^{a} ± 2.83	38.12^{c} ± 1.64	43.09^{b} ± 1.40	35.58^{d} ± 3.24	<0.001
	Cost/WD	68.60^{b} ± 15.06	170.09^{a} ± 83.61	58.83^{b} ± 8.22	78.18^{ab} ± 24.75	<0.001
	Income	-547.13^{ab} ± 383.26	-939.30^{b} ± 468.58	-146.07^{a} ± 236.43	-153.66^{a} ± 515.34	0.002
P1	Cost (MR&SM)	3342.20^{a} ± 156.56	2757.75^{c} ± 61.59	2957.23^{b} ± 82.67	2423.74^{d}± 132.99	<0.001
	Cost (MR)	3037.05^{a} ± 62.53	2636.14^{b} ± 55.97	2593.38^{b} ± 48.94	2180.29^{c} ± 62.00	<0.001
	Cost (SM)	305.15^{ab} ± 132.50	121.61^{c} ± 66.06	363.85^{a} ± 71.09	243.45^{b} ± 136.18	<0.001
	ADC	53.05^{a} ± 2.49	43.77^{c} ± 0.98	46.94^{b} ± 1.31	38.47^{d} ± 2.11	<0.001
	Cost/WD*	92.81^{b} ± 27.73	247.71^{a} ± 151.14	77.43^{b} ± 10.09	115.04^{ab} ± 62.96	0.001
	Income	-840.41^{b} ± 328.70	-1031.43^{b} ± 397.40	-459.05^{a} ± 148.08	-293.88^{a} ± 463.84	<0.001
P2	Cost (MR&SM)	334.13^{a} ± 74.85	177.79^{b} ± 110.00	360.47^{a} ± 41.24	315.67^{a} ± 121.75	0.002
	Cost (SM)	334.13^{a} ± 74.85	177.79^{b} ± 110.00	360.47^{a} ± 41.24	315.67^{a} ± 121.75	0.002
	ADC	23.87^{a} ± 5.35	12.70^{b} ± 7.86	25.75^{a} ± 2.95	22.55^{a} ± 8.70	0.002
	Cost/WD	**19.49 ± 2.79**	*** 25.41 ± 16.06**	**19.83 ± 3.61**	**26.46 ± 11.78**	**0.384**

^{a,b,c,d} Means within row with different superscripts differ significantly ($P < 0.05$).

* As a calf from group B lost weight during P2, FCR for this calf could not be calculated and the value was removed before statistical analysis was performed, this will also be the case for Cost/WD for P2. The statistical analysis was therefore done with a missing value (n = 7). It can be presumed that the mean and standard deviation for this group should be larger than the reported values.

Cost was calculated as the amount spent per phase for milk replacer and starter meal and is therefore specific to this trial period. In different commercial systems these periods may differ depending on specific rearing conditions and practices. Further, the external market forces which can affect profitability parameters (Cortéz & Manual, 2010) of different dates and time have not been accounted for in the current study. Average daily cost (ADC) gives an indication of the cost per day, and this is a useful tool when managing a calf unit and for the development of budgets. Cost and ADC will therefore follow the same trend.

Since milk replacer intake was regulated, intake did not contribute to the differences in milk replacer cost, and it is therefore a function of milk replacer price. Regarding milk replacer cost, A (Biomel) had the highest cost at R 3037.05 and D (FP-Kalpap) had the lowest cost at R2180.29 ($P < 0.05$).

The cost of B (FP-Biomel) and C (Kalfpap) did not differ from each other but differed ($P < 0.05$) from A and D (Table 4.4).

As the same starter meal was used for all the treatments (Table 4.2), differences in starter meal cost were determined by variance in intake. These values follow the same trend as DMI (Chapter 3, Table 3.5). In P1 A and C had the highest cost and in P2 A, C and D had the highest cost. The lowest cost in P1 was D at R243.45 and in P2 was B at R177.79. As a higher intake is advantageous as it leads to improved growth the lower cost is not necessarily favourable.

When considering the milk replacer and starter meal in combination in TP, A and C had the best FCR (Chapter 3, Table 3.5), and A had the highest ($P < 0.05$) cost at R 3676.33/calf with an ADC of R47.74/calf. Although D had the lowest ($P < 0.05$) cost at R2739.40/calf and ADC of R 35.577/calf it had the poorest FCR with B (Chapter 3, Table 3.5). Thus, when considering both milk replacer and starter meal cost for the TP and not compromising on FCR C with a cost of R3317.70/calf and an ADC of R43.09/calf should be considered. This would be the best FCR for the lowest cost considering both milk replacer and starter meal intake in this trial. The FCR is an important parameter when determining the financial viability of a specific milk replacer as this gives an indication of the DMI needed per kg weight gain (Hanel, 2020).

A cost benefit analyses considers both growth performance as well as the cost incurred (O'Mahony, 2021). Cost per weight difference (Cost/WD) and income are the reported parameters that take both growth performance and cost incurred into account. As income is dependent on the weight of the animal and therefore growth rate (given the starting weight is similar), this parameter provides the best indication of the efficacy of a specific feeding regime. As shown in Figure 4.2 below, cost per weight difference is the feed conversion multiplied by the feed cost per kg, and this provides a useful way to compare different diets and feeding regimes. This methodology provides an indirect way to calculate cost benefit, while also taking performance of the calves into account. This is the profit made when selling a calf after feeding cost and the calf purchase price (R360) is subtracted.

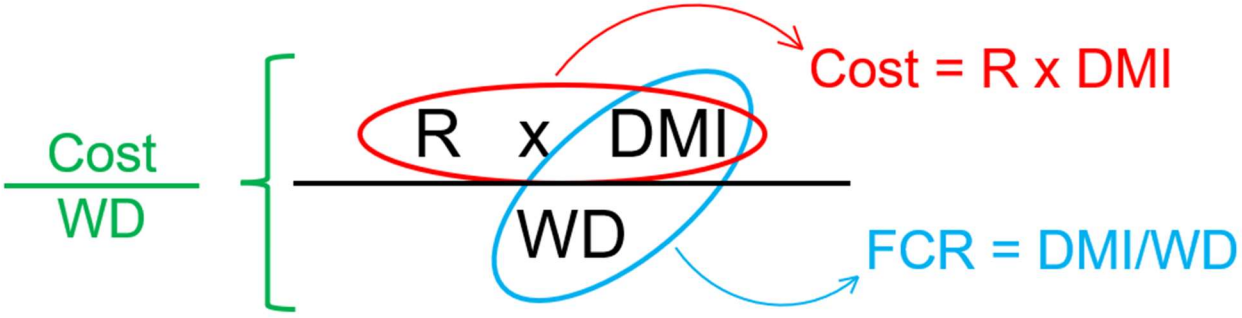

Figure 4.2 The way in which Cost/WD is calculated referred to as cost of gain by Albright *et al.* (1993).

One of the calves in group B lost weight during P2 and as FCR renders a negative result with weight loss it can only be calculated when a positive growth rate is attained. Under these circumstances

this will also result in a negative Cost/WD. The data for this calf was therefore not used when calculating the profitability parameters. Although the P-value indicated that there was no difference during P2 amongst treatments (Table 4.4), this may be due to the duration of P2 which may have been too short for differences to develop to the extent where any meaningful differences could develop. Therefore, the influence of the P2 is more prominent during TP. During both P1 and TP significant differences for Cost/WD were observed between the different groups (Table 4.4). As TP covers the whole trial period Cost/WD of this phase is the best parameter to use and in terms of this parameter, C (Kalfpap) was the most efficient although the treatment did not significantly differ from A (Biomel) and D (FP-Kalfpap). This can also be ascribed to the relatively large variation (Table 4.4) within groups.

It is important to note that the income calculation relates to the experimental conditions and not commercial circumstances and that the main purpose of the trial was to investigate the influence of carbohydrate levels and protein sources in milk replacers. As was discussed in Chapter 3 the income calculations may not be directly applicable to large-scale commercial units. In these units weaning generally forms part of an extended production process and although cost is carefully managed, profit is mostly generated by weight gain during the back grounding phase where the weaned calves are normally fed grazing until they reach a weight of between 220 and 240 kg before they are placed into a feedlot (Blom *et al.*, 2022). Therefore, the trends are arguably more important for this parameter than the actual values.

When calculating income, the P2 value only takes a 2-week observation perioded in consideration and the only value of this is to have some insight towards the extent of weaning shock and the resultant effect on body weight change.

If the calves had been sold on day of weaning the data represented during P1 for C (Kalfpap) and D (FP-Kalfpap) showed higher ($P < 0.05$) income compared to A (Biomel) and B (FP-Biomel). Considering TP, groups A, C and D provided the highest ($P < 0.05$) income, and B had the lowest income for this phase, but it did not differ from A (Table 4.5).

4.5 Conclusion

For the total trial period, treatment D emerged as the most cost-effective when considering only cost and average daily cost (ADC) (Table 4.4). However, when also considering feed conversion ratio (FCR) for the lowest cost, treatment C proved superior. This result was also echoed by both income and cost per weight difference (Cost/WD). Although treatment C had the lowest Cost/WD, it did not differ from treatments A and D. Similarly, the income of treatment C showed the highest return, but also did not differ from A and D.

Overall, the data suggest that treatment C might be the most advantageous under the specific trial conditions. However, if the recommendations for improving feed processing (fermented protein) and

reducing trypsin inhibitor content to below 4 mg/g protein can be achieved, different profitability results might become evident.

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Chapter 5
Body weight estimation of pre-weaned Holstein bull calves

5.1 Abstract

Girth circumference weight estimating tapes is a practical and cost-efficient way to easily predict the weight of calves. Although the accuracy of prediction is influenced by factors such as breed, sex and whether the animal is pre- or post-wean, accuracy can be optimized by using a tape tailor made for a specific breed and physiological stage of the animals. When developing girth circumference weight estimating tapes, meta-studies should be used and it is expected that the data collected during this study would form part of a larger data base from multiple studies. This can then be used to improve the accuracy of a girth measuring tape or an algorithm for calculating expected weight of Holstein calves in South Africa. In this study, data was collected from a total of 32 Holstein bull calves divided in 4 groups (n=8) and fed different milk replacers. The trial period was divided into two phases. Phase 1 (P1, days 0–63), during which milk replacers were fed, and Phase 2 (P2, day 63-77), a two-week post-weaning period. During the whole trial period starter meal was available *ad libitum* and all intakes and refusals were recorded. Calves were measured and weighed once a week.

The specific measuring tape used in the current study overestimated the weight of pre-weaned Holstein bull calves by 10 kg for calves with a girth circumference of between 70 and 80 cm and this deviation increased with approximately 1 kg for each additional 10 cm increase in girth circumference over 80 cm. A specific measure tape for juvenile dairy animals to allow more accurate weight prediction needs to be developed.

5.2 Introduction

Birth weight data can assist dairies to make better decisions about calf rearing and gestating cow nutritional management strategy. Live weight gain is used for managing feeding regimes (Curtis *et al.*, 2018; Dingwell *et al.*, 2006) and determination of the dosage rate of medications, including antibiotics and anthelmintics (Enevoldsen & Kristensen, 1997; Dingwell *et al.*, 2006; Machila *et al.*, 2008; Van Dijk *et al.*, 2015).

The most accurate way to determine the body weight of a calf is to use an electronic scale. The use of an electronic scale however has a cost implication to farmers, and this is often beyond the means of many farmers. It can also be time-consuming if there is a lack of infrastructure. For this reason, it might not be the preferred method even on farms that do have access to an electronic scale (Heinrichs *et al.*, 1992; Dingwell *et al.*, 2006).

In response to the above reasons, methods have been developed to estimate the weight of a calf without the use of an electronic scale. One of these methods is the use of a measuring tape (Dingwell

et al., 2006). The use of a measuring tape allows the farmer to estimate the weight of cattle without being restricted by the lack of portability of a scale and although there is handling involved, it's easy to learn and apply. The measuring system exploit the high relationship between girth circumference of calves and their weight (Wangchuk et al., 2017).

Currently there is no single standard with regards to measuring tapes and the predicted weight when using different tapes vary considerably (Heinrichs et al., 2007). This result is influenced by several factors and the specific algorithm fitted to the data used when developing the specific measuring tape (Ruchay et al., 2022). Commonly used tapes include tapes that are used to measure the girth and hoof circumference, and studies have been conducted to compare the two methods as well as comparing the predicted weights using these methods with scale weight (Sharpe & Heins, 2023). The girth tape is marked in centimetres (or another measuring unit) and predicts the corresponding body weight for each increment. The predicted weight is derived from a model developed by measuring the weight and girth of many cattle. The girth tape measures the chest circumference of the body behind the front shoulder. The hoof girth tape measures the circumference of a specific hoof of the calf and the weight is then predicted using this parameter.

Heinrichs & Hargrove (1987) measured 5,723 heifers on commercial farms in Pennsylvania and reported a good correlation between heart girth circumference and body weight.

Age of calves appears to have an influence on the accuracy of prediction when using a heart girth tape. Dingwell *et al.* (2006) reported a significant difference between scale weight and heart girth tape in calves younger than three months and that heart girth tape measurement was significantly lower than the scale measurements. It is important to note that this period is mostly during the pre-weaned phase and that the diet during this period largely consists of milk and milk replacers. Dingwell *et al.* (2006) further recommends that the study of the heart girth tape for predicting the weight of very young Holstein calves should be investigated further.

Breed is presumed to influence the accuracy of predictions when using the heart girth tape and some studies suggest that better results are obtained for certain breeds when using specific tapes (Conan *et al.*, 2018). Sharp & Heins (2023) report that that accuracy has been confirmed to a larger degree in some purebred dairy breeds, but that the use of heart girth tapes and hoof-circumference tapes may not be accurate in estimating the weight of crossbred calves.

Sex does not appear to have a specific influence on accuracy of weight predictions when using heart girth tapes, although according to Odadi (2018), these measurements were taken in sexually immature animals, and this may not hold true for older animals.

The aim of the study was to evaluate the use of a commercial (Nandrea Health Products, Oudtshoorn, South Africa) girth circumference measuring tape to predict the body weight of juvenile Holstein dairy calves. Data of the current study would contribute to the development of accurate body weight prediction technology.

5.3 Materials and methods

Ethical clearance was obtained from the UFS Animal Research Ethics Committee for all trial related practices (UFS-AED2023/0051).

Data of the current study originated from the larger study presented in Chapter 3 and 4. For clarity a brief explanation on the feeding regime is provided (Appendix A, Table A.1). It is however important to note that the measurements were taken from pre-weaned Holstein bull calves.

During the trial a regime of 3 feedings of 2 litre milk replacer per day was used. After weaning the calves were kept in the unit and observed for 2 weeks. Any milk replacer refusals were noted. A standard commercial starter meal was used, and the calves had *ad libitum* access to it throughout the trial. Weekly starter intake was determined by weighing feed offered and refusals.

Thirty-two Holstein bull calves were sourced from a local farmer and used in the trial. These calves received colostrum after birth and entered the trial at an age of between 3 and 4 days. The calves were marked and numbered with different colour tags and randomly allocated to treatment groups. These groups are not relevant to this trial as the parameters used were girth circumference and scale weight

A standard vaccination program was followed, and calves were weighed and measured on a weekly basis. The duration of the trial was 77 days and calves were weaned on day 63. Each calf was therefore weighed and measured 12 times as the calves were also weighed and measured at the start of the trial. Although the calves were weighted at weekly intervals, time was not used as a parameter when determining predicted weight and only girth circumference was used.

The trial data was grouped according to the different periods as depicted in Figure 5.1. Phase 1 (P1) refers to the period during which the calves were fed milk replacer and was from day 0 – 63. Day 63 was also the day on which the calves were weaned. Phase 2 (P2) was the two-week observation period after weaning, starting on day 64 and terminating on day 77. This phase was used to determine the level of wean shock. The total phase (TP) started on day 0 and concluded on day 77. The data of the two-week post-weaning period also serves a practical purpose as calves are often sold directly after weaning and under those circumstances a more accurate determination of weight is an advantage.

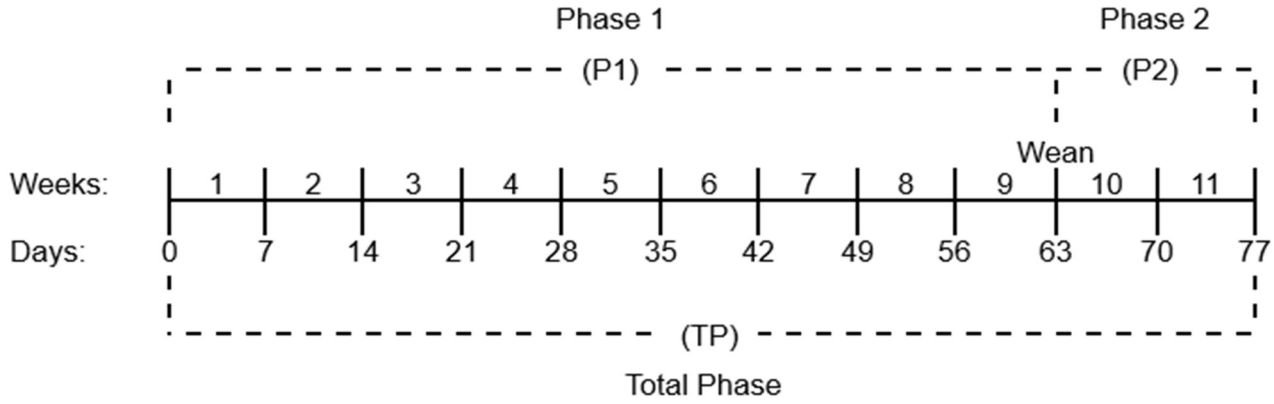

Figure 5.1 A line diagram of the time periods used in the trial. Calves were weighed, and measured on a weekly basis, starting on Day 0.

The different milk replacers used were formulated on an iso-caloric and iso-nitrogenous basis and random milk replacer and starter feed samples were taken from different batches throughout the trial and the pooled samples were analysed by a proximate analysis. The protein content was determined according to standard methods prescribed by AOAC (2002) using the Dumas method and a LECO FP828 (St Joseph, MI, USA). The fat in the milk replacers was determined by Assurecloud using a gravimetric method (Evers *et al.*, 2000), because fats in milk replacers cannot be accurately determined by a proximate analysis (ether extract) as it does not efficiently extract emulsified fats (Moneeb *et al.*, 2021). The average metabolic energy of the milk replaces was 12.44 MJ/kg and had an average crude fat of 136.6 g/kg. The average nitrogen content was 32.2 g/kg which implies an average crude protein content of 201.25 g/kg. The starter meal had a nitrogen content of 24.7 g/kg and thus a crude protein content of 154.4 g/kg, with a metabolizable energy of 10.57 MJ/kg. The crude fat content tested at 3.17 g/kg and this is in line with the general guidelines for ruminating cattle (Fiorentini *et al.*, 2015; Bionaz *et al.*, 2020).

Girth circumferences that correlated to a predicted weight was collected 12 times during the trial period for all 32 Holstein bull calves after a scale weight was taken. The predicted weight was linearly correlated to scale weight. The correlation line gives the values that a calf is most likely to weigh for a specific measure.

The coefficient of determination (R^2) was calculated to determine the fit of the model. The coefficient of determination is a measure that provides information about the goodness of fit of a model and is a statistical measure of how well the regression line approximates the actual data. Thus, R^2 is a statistical measure in a regression model that determines the proportion of variance in the dependent variable used to determine the independent variable.

5.4 Results and Discussion

The girth circumference tape investigation was performed to access the accuracy of prediction of a commercial girth circumference tape (Nandrea Health Products) for Holstein bull calves. It should however be noted that under normal circumstances a meta data set would be required for more accurate results.

Figure 5.2 is a plot of the scale weights as it correlates to a specific predicted weight according to the girth circumference measurement. It gives a visual overview of how calf scale weights varied for a specific girth circumference measurement. The weight determined by girth circumference is referred to as predicted weight.

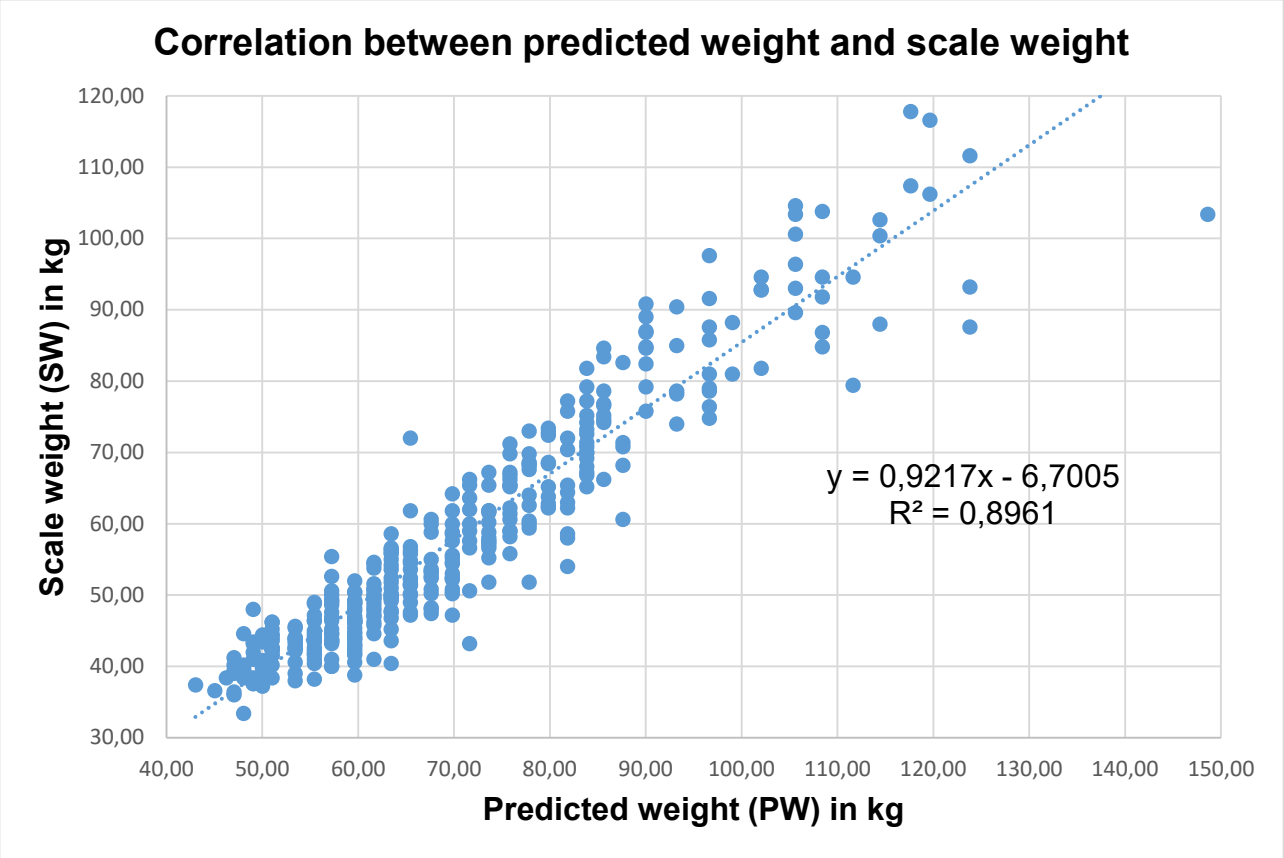

Figure 5.2 Correlation of predicted weight determined by a commercial girth circumference tape and the scale weigh of neonatal Holstein bull calves.

Compounded values for intervals of approximately 10 cm on the measuring tape have been calculated to be able to make corrections when using the specific commercial (Nandrea Health Products) measuring tape when estimating the weight of pre-weaned Holstein bull calves.

According to the coefficient of determination (R^2), the independent variable and predicted weight (derived from girth circumference) explain 89,61% of the variation in the target variable, scale weight.

As mentioned, the correlation line gives the values that a calf is most likely to weigh for a specific measure. It is however important to note the predicted weight is plotted and not girth circumference on the X axis although there is a girth circumference that corresponds to predicted weight. Therefore,

when using the equation, y = 0,9217x-6,7005 the girth circumference should first be converted to predicted weight and then used as the x value in the equation.

Table 5.1 Corrections to Nandrea Health Products measuring tape when estimating weight for pre-weaned Holstein bull calves. Predicted weight (PW) was determined by calf girth circumference (GC) and correlated to scale weight (SW).

GC measuring tape (cm) range	Average GC measuring tape (cm)	Estimated PW	Average SW	Calculated SW (y = 0,9217x-6,7005)	Difference from PW
70-80	77.667	44.733	37.467	34.533	10.20
80-90	84.500	53.220	43.020	42.357	10.86
90-100	94.500	72.660	59.137	60.276	12.38
100-110	104.500	95.180	83.413	81.035	14.15
110-120	112.000	117.400	101.093	101.516	15.88
120-130	122.000	148.600	103.400	130.276	18.32

The results in Table 5.1 clearly indicate that the specific commercial measuring tape overestimates the weight of pre-weaned Holstein bull calves, although it would need a much larger data set to improve the reliability of results.

The commercial girth circumference measuring tape used in the current study was originally developed using data obtained from studies with Jersey calves. As Holsteins are generally heavier with a larger body frame and both their pre-weaning and post-weaning weight at the same age tend to be heavier than that of Jersey calves. It appears therefore that the differences in body frame between the breeds influenced the accuracy of correlation between measured girth circumference and predicted body weight. It is thus possible that the accuracy of prediction when using the same girth circumference measuring tape for both breeds would vary (Sloniewski *et al*., 2005; Grimwood *et al.*, 2023). The measuring tape values and the corresponding predicted weight is summarised in Appendix A (Table A.6 and A.7).

The pre- and post-weaning period influence the accuracy of weight prediction when using a girth circumference tape, not because the growth rate differs but because the expansion of the rumen influences girth circumference. The rumen increases from 30 to 70% of the total capacity of the gut during the weaning process (Warner *et al.*, 1956; Baldwin VI *et al.*, 2004). This is however counteracted by the fact that dairy calves tend to start taking in significant amounts of dry matter at the same age, indicating rumen development, and are generally weaned at the same age range (Eckert *et al*., 2015; Palczynski *et al*., 2020). Measurement of calves using different rearing systems and at different weight when compiling the database will therefore partially compensate for this.

It is thus concluded that the most important factor to be considered when calibrating a girth circumference tape is breed. Although many factors including liquid of solid feed phase, environment

and sex influence the weight of the calf, it does not necessarily change the relationship between the girth circumference and weight of the animal.

5.5 Conclusion

Although the sample used was not large enough to accurately determine the accuracy of weight prediction of the commercial girth circumference tape used in this study, the data can contribute to a later meta-study. It further gives a useful indication that this commercial girth tape overestimates the weight of pre-weaned Holstein calves as well as calves in the first 2 weeks after weaning. The measuring tape used overestimated the weight of pre-weaned Holstein bull calves by 10 kg for calves with a girth circumference of between 70 and 80 cm and this deviation increased with approximately 1 kg for each additional 10 cm.

For a more accurate prediction of weight using a girth circumference tape, the tape will have to be calibrated using a larger data set of a specific breed and feeding regime.

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Chapter 6
General Conclusions and Recommendations

6.1 General Conclusions

The global milk replacer market is experiencing significant growth, driven by rising demand for dairy and meat products worldwide. The South African market, although a very small fraction of the global market, has followed the same trend. However, the high cost of raw materials used for formulating milk replacers is decreasing the economic viability of using milk replacers to rear calves, especially for bull calves reared for the meat industry and to a lesser extent for replacement heifers. This presents a challenge and is forcing the industry to investigate less costly high quality alternative raw materials. This study aimed to address this issue by investigating the biological viability (Chapter 3) and cost-effectiveness (Chapter 4) of alternatives to traditional milk replacers, focusing on the use of fermented plant proteins and high carbohydrate inclusion as raw materials in milk replacers. In Chapter 5 the issue of economic viability is further addressed by investigating the effectiveness of using a commercial girth tape to predict weight as an alternative to the use of a more costly electronic scale.

The study reported in Chapter 3 was designed to assess the biological viability of milk replacers formulated to include fermented plant protein and a higher level of carbohydrates. Fermented plant proteins are considered as a potential alternative to expensive animal proteins, while high carbohydrate content was explored as an alternative energy source and as a means to reduce wean shock by stimulating starter meal intake without compromising calf growth and health. In this study the 4 treatments were a conventional milk replacer, one containing 20% fermented protein, a high carbohydrate milk replacer and a high carbohydrate milk replacer containing 20% fermented protein. During the 77-day trial, growth rates, feed efficiency and dry matter intake were recorded. The study also assessed the carry-over effects of these formulations post-weaning and the incidence of wean shock. The results indicated that fermented protein containing milk replacers performed poorly due to the high levels of trypsin inhibitors, which hindered digestibility and overall growth. As a result, the study concluded that unless fermented protein is processed to reduce trypsin inhibitor levels below 4 mg/g protein, it should not be included in milk replacers. In contrast, high carbohydrate milk replacers, when introduced after a proper transition period, showed growth performance on par with conventional milk replacers and stimulated starter meal intake. This highlighted the potential for high carbohydrate formulations as alternatives for calf rearing.

In Chapter 4 the focus shifted to the financial aspect and here the focus was on the financial implications of using high carbohydrate and fermented plant protein-based milk replacers. While biological performance is essential, the cost-effectiveness of these alternatives is crucial for their widespread adoption, particularly in low-resource regions where farmers face financial constraints.

The chapter examined the cost of production, feed conversion rate, and profitability parameters of various milk replacer formulations. It was hypothesized that reducing the cost of milk replacers could make them more accessible to small-scale farmers without compromising the health and growth of the calves. Results from the financial analysis indicated that high carbohydrate milk replacers were the most cost-effective when considering feed conversion ratio (FCR) and income per weight gain. This treatment also had the lowest total cost and the best return on investment, although it did not significantly outperform other formulations like the conventional milk replacer and fermented protein based high carbohydrate milk replacer. The study also suggested that if improvements in the processing of fermented plant protein were made, it could become a more cost-effective option in the long term.

Chapter 5 expands on one of the general aims of the study in that it investigates a way in which the economics of calf rearing can be improved. Accurate weight determination is essential for managing feeding regimes, medication dosages, and overall farm management. While electronic scales provide reliable weight data, they are often prohibitively expensive for small-scale farmers. The chapter investigated the potential of using a commercial girth circumference measuring tape as an affordable alternative for estimating calf weight with the view to start the ongoing accumulation of data. This was done to enable the establishment of a larger data basis in future which could be used to improve the prediction of weight using a girth circumference tape for dairy calves in South Africa. The study tested a specific commercial measuring tape to evaluate its accuracy in predicting the weight of pre-weaned Holstein bull calves. Results showed that the specific tape overestimated the weight of calves, with girth circumferences between 70 and 80 cm, by about 10 kg and approximately another kg of deviation for each additional 10 cm increase in girth circumference. The deviation in weight estimation increased with the size of the girth circumference. The study concluded that, while girth circumference measuring tapes could provide a cost-effective alternative, further calibration and refinement are necessary to improve their accuracy. Specifically, the tape needs to be adjusted for different calf breeds and developmental stages to ensure more reliable weight estimates. Despite the need for improvement, the use of such measuring tapes offers a promising, low-cost tool for farmers, especially in regions where electronic scales are not feasible.

In conclusion, the study highlighted the potential for high carbohydrate milk replacers to reduce costs while maintaining calf health and performance. The findings suggest that further research is needed to confirm that the use of high carbohydrate milk replacers stimulates starter meal intake both before and after weaning.

6.2 Recommendations

The intent of the study was to investigate the effect on growth performance and profitability of Holstein bull calves when feeding a milk replacer containing fermented plant protein and/or a milk replacer with a high carbohydrate content. During the trial multiple question arose as understanding

improved and this has led to the following recommendations. The relevant section of the thesis providing background information in this regard is referred to.

In Chapter 3 Section 3.4.4.5 Discussion, under the main effect of protein the influence of trypsin inhibitor in milk replacer were described. Pre-ruminant animals seem to be more sensitive to the inclusion of trypsin inhibitor than mature ruminants as the degradation in the rumen is not present. However, there is little research on the effect of specific levels of trypsin inhibitor in milk replacers for pre-ruminants. A study on the influence and consequences of different trypsin inhibitor levels would add value and could potentially lead to the development of new affordable raw materials for use in milk replacers. As it was clear that the levels of trypsin inhibitor present in the milk replacer containing fermented protein depressed calf performance, different ways in which this problem can be addressed were discussed. It was concluded that if a viable solution could be found, fermented protein can potentially still be profitable option for inclusion in milk replacers. However, it is recommended that a new trial is conducted with the improved protein sources to verify this hypothesis.

In Chapter 3 Section 3.3.3.6 Discussion, the influence of high carbohydrate milk replacers on the stimulation of starter meal was discussed. It was concluded that due to the limited period the effect could not be fully determined, and it is recommended that this should be further investigated to verify this observation and determine the carry-over effect during the back-grounding phase and even during the feedlot phase.

In this trial the feed conversion for the milk replacers and starter meal could not be calculated independently and it was suggested that a trial could be designed to determine the contribution of the milk replacer and starter meal. As this would only be valid under specific conditions it was concluded that due to the limited application it is doubtful if this would be worth pursuing. One of the limitations experienced during this trial was that although dry matter intake could be divided between milk replacer dry matter intake and starter meal dry matter intake, feed conversion ration could only be calculated for total dry matter intake as the growth attributed to milk replacer or starter meal cannot be easily separated. During this phase the calf relies to a large degree on enzymatic digestion in the abomasum, but over time the importance of ruminal digestion through fermentation increases. It should be possible to determine the efficiency of feed conversion of the starter meal if 2 groups of calves are used and both groups of calves are fed a standardised quantity of milk replacer and one of the groups is fed a determined additional weight of starter meal. The difference in weight gain between the 2 groups could then be attributed to starter meal intake and efficiency of feed conversion at different ages for starter meal could be calculated. This may be a refinement of these calculations but might not be practical as the results would only be applicable for a specific set of conditions and would not be applicable under other circumstances.

In Chapter 5 the verification of a commercial weight predicting girth circumferences tape was investigated. The results indicate that for more accurate prediction of weight the tape will have to be

calibrated for a more narrowly defined group of animals, taking breed and whether the calves are in the pre- or post- weaning phase into account. Despite the relative sample size, making meaningful conclusions difficult, the data is still a useful contribution as it is envisaged that it can form part of a larger data set in future. This larger set of data could then lead to meaningful use and application.

Appendix A
Trial outline, Procedure and Collected data

The trial procedure (Table A.1) used in chapter 3, 4 and 5. In chapter 5, calf girth circumference was measured on a weekly basis. A standard vaccination program was followed but is not included as this may differ depending on the location. It would be best to consult a local veterinarian for a vaccination program applicable for the specific location. Calves for this trial were vaccinated for pneumonia using a nasal spray, as prescribed by the veterinarian (2 ml Inforce 3®, half the dose (1 ml) in each nostril of the calve on day 5).

Table A.1 The 77-day trial outline for 32 Holstein bull calves on four different milk replacers treatments (A, B, C and D). It is also indicated when weight, measurements and intake were recorded.

Before trial	Calves were suckled by dam to ensure adequate colostrum intake Calves were transported to trial site.			
Day 0	Calves were measured, weighed and randomly allocated to one of the treatments and pens. (Calves were weighed weekly from this day.) The time of arrival at the unit determined how many feedings the calve received (1 litre per feeding).			
Day 1	Commercial milk replacer (Standard) Biomel® (SP SC) **A** 16 calves 1 litre per feeding, 3 feedings per day Start *ad lib* calf starter meal	Milk replacers containing fermented protein FP-Biomel® (FP SC) **B** 16 calves		
Day 2	1.2 litre per feeding, 3 feedings per day Daily feed refusals weighed			
Day 3	1.4 litre per feeding, 3 feedings per day Daily feed refusals weighed			
Day 4	1.6 litre per feeding, 3 feedings per day Daily feed refusals weighed			
Day 5	1.8 litre per feeding, 3 feedings per day Daily feed refusals weighed			
Day 6	2 litre per feeding, 3 feedings per day Daily feed refusals weighed			
Day 7	2 litre per feeding, 3 feedings per day Daily feed refusals weighed **Measure and weigh calves** (Weekly weigh) Castration by banding (Elastic)			
Day 8-10	2 litre per feeding, 3 feedings per day Daily feed refusals weighed			
Day 11	Commercial milk replacer (Standard) Biomel® (SP SC) **A** 8 calves	Milk replacers containing fermented protein FP-Biomel® (FP SC) **B** 8 calves	High carbohydrate milk replacers Kalfpap® (SP HC) **C** 8 calves	High carbohydrate milk replacers containing fermented protein FP-Kalfpap® (FP HC) **D** 8 calves

	Daily feed refusals weighed Start adaption to high carbohydrate milk replacers (HCMR). Treatment groups on HCMR (C&D); ↳ A:C = 3:1 = 1.5 litre : 0.5 litre ↳ B:C = 3:1 = 1.5 litre : 0.5 litre 2 litre per feeding, 3 feedings per day
Day 12	Daily feed refusals weighed Adaption to high carbohydrate milk replacers (HCMR). Treatment groups on HCMR (C&D); ↳ A:C = 1:1 = 1 litre : 1 litre ↳ B:C = 1:1 = 1 litre : 1 litre 2 litre per feeding, 3 feedings per day
Day 13	Daily feed refusals weighed Adaption to high carbohydrate milk replacers (HCMR). Treatment groups on HCMR (C&D); ↳ A:C = 1:3 = 0.5 litre : 1.5 litre ↳ B:C = 1:3 = 0.5 litre : 1.5 litre 2 litre per feeding, 3 feedings per day
Day 14	2 litre per feeding (the adaption to high carbohydrate milk replacers (HCMR) completed), 3 feedings per day Daily feed refusals weighed **Measure and weigh calves** (Weekly weigh)
Day 15-58	2 litre per feeding, 3 feedings per day Daily feed refusals weighed Weekly **measure and weigh calves** (Day 21, 28, 35, 42, 49, 56)
Day 59-60	2 litre per feeding, 2 feedings per day Daily feed refusals weighed
Day 61-62	1 litre per feeding, 2 feedings per day Daily feed refusals weighed
Day 63	1 litre per feeding, 2 feedings per day Daily feed refusals weighed **Measure and weigh calves** (Weekly weigh)
Day 64-77	Observation period (To determine the effect of weaning) Daily feed refusals weighed Weekly **measure and weigh calves** (Day 70, 77)
Day 78	Calves leave system

Table A.1 correlates to the line diagram in chapter 3 (Figure 3.1), 4 (Figure 4.1) and 5 (Figure 5.1). These figures depict the timeline of the trial and indicate at which points data was collected.

Due to practical experiences during the trial, it is recommended that for future calf rearing trials the procedure is adjusted if a similar trial is performed. Table A.2 incorporate the suggested adjustments. It was noted that calves adjust better with less digestive disturbances if lower initial volume are fed. Improved results are obtained if elastic is applied at an older age when testicular development is a bit more advanced. Although milk replacer intake and refusals should be noted on a daily basis, it is easier from a management perspective to determine weekly stater meal intake and as the animals are only weighed and measured on a weekly basis the additional data for daily intake do not add value.

Table A.2 The adjusted 77-day trial outline for 32 Holstein bull calves on four different milk replacers treatments (A, B, C and D). It is also indicated when weight and intake were recorded.

Before trial	Calves were suckled by dam to ensure colostrum intake Calves were transported to trial site.			
Day 0	Calves were weighed and randomly allocated to treatments and pens. (Calves weighed weekly from this day.) The time of arrival at the unit determined how many feedings the calve received (1 litre per feeding).			
Day 1	Commercial milk replacer (Standard) Biomel® (SP SC) **A** 16 calves		Milk replacers containing fermented protein FP-Biomel® (FP SC) **B** 16 calves	
	1 litre per feeding, 3 feedings per day Start *ad lib* calf starter meal			
Day 2-4	1.2 litre per feeding, 3 feedings per day			
Day 5-6	1.4 litre per feeding, 3 feedings per day			
Day 7	1.4 litre per feeding, 3 feedings per day Weekly feed refusals weighed **Weigh calves** (Weekly weigh)			
Day 8-10	1.6 litre per feeding, 3 feedings per day			
Day 11	Commercial milk replacer (Standard) Biomel® (SP SC) **A** 8 calves	Milk replacers containing fermented protein FP-Biomel® (FP SC) **B** 8 calves	High carbohydrate milk replacers Kalfpap® (SP HC) **C** 8 calves	High carbohydrate milk replacers containing fermented protein FP-Kalfpap® (FP HC) **D** 8 calves
	Start adaption to high carbohydrate milk replacers (HCMR). Treatment groups on HCMR (C&D); ↳ A:C = 3:1 = 1.35 litre : 0.45 litre ↳ B:C = 3:1 = 1.35 litre : 0.45 litre 1.8 litre per feeding, 3 feedings per day			
Day 12	Adaption to high carbohydrate milk replacers (HCMR). Treatment groups on HCMR (C&D); ↳ A:C = 1:1 = 0.9 litre : 0.9 litre ↳ B:C = 1:1 = 0.9 litre : 0.9 litre 1.8 litre per feeding, 3 feedings per day			
Day 13	Adaption to high carbohydrate milk replacers (HCMR). Treatment groups on HCMR (C&D); ↳ A:C = 1:3 = 0.45 litre : 1.35 litre ↳ B:C = 1:3 = 0.45 litre : 1.35 litre 1.8 litre per feeding, 3 feedings per day			
Day 14	2 litre per feeding (the adaption to high carbohydrate milk replacers (HCMR) completed), 3 feedings per day Weekly feed refusals weighed **Weigh calves** (Weekly weigh) Castration by banding (Elastic)			
Day 15-58	2 litre per feeding, 3 feedings per day Weekly feed refusals weighed (Day 21, 28, 35, 42, 49, 56) Weekly weigh - **Weigh calves** (Day 21, 28, 35, 42, 49, 56)			
Day 59-60	2 litre per feeding, 2 feedings per day			
Day 61-62	1 litre per feeding, 2 feedings per day			
Day 63	1 litre per feeding, 2 feedings per day			

	Weekly feed refusals weighed **Weigh calves** (Weekly weigh)
Day 64-77	Observation period (To determine the effect of weaning) Weekly feed refusals weighed (Day 70, 77) Weekly weigh - **Weigh calves** (Day 70, 77)
Day 78	Calves leave system

Table A.3 The weekly scale weight (kg) of 32 Holstein bull calves over a 77-day trial with four milk replacer treatments (A, B, C and D).

Treatment and calves		Days											
		0	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**	**70**	**77**
Biomel (A)	**1**	38.4	39.8	45.0	50.6	53.4	57.6	67.6	75.8	84.8	94.6	104.6	117.8
	2	40.2	44.6	46.8	51.6	57.6	60.0	68.2	76.6	81.8	92.8	103.4	116.6
	3	44.6	50.0	51.6	55.8	58.2	62.2	68.4	74.2	78.6	89.0	93.0	103.4
	4	36.6	33.4	41.0	46.2	49.6	55.6	61.8	65.4	70.8	79.4	88.0	97.6
	5	40.2	41.6	45.4	45.4	47.0	50.0	54.5	58.0	60.6	68.6	75.2	86.8
	6	42.6	45.2	47.2	50.2	54.2	55.2	58.8	60.2	66.2	72.0	79.0	85.0
	7	38.6	41.6	43.6	47.4	50.8	57.4	61.4	70.4	74.6	76.4	81.0	89.6
	8	37.6	40.2	38.4	37.6	38.2	44.0	46.4	50.2	54.4	59.0	66.8	74.0
FP-Biomel (B)	**9**	38.4	47.8	50.2	47.8	47.2	43.6	45.2	38.8	43.2	43.8	47.6	51.4
	10	43.8	46.6	51.8	50.4	50.6	51.8	53.0	64.0	69.8	68.4	75.8	81.0
	11	48.2	52.4	54.6	59.0	59.4	61.8	63.0	65.4	62.2	65.2	66.2	71.4
	12	39.8	42.6	43.2	46.4	51.8	56.4	58.8	65.4	69.2	77.2	84.6	94.6
	13	39.0	42.6	41.8	43.6	43.6	41.0	42.6	44.0	47.2	50.2	56.6	52.4
	14	45.2	49.0	49.8	50.8	50.0	50.2	52.4	52.2	55.2	57.0	62.2	56.6
	15	38.4	42.6	43.4	41.6	40.6	38.0	40.0	40.0	41.8	44.6	49.8	48.2
	16	40.4	41.6	43.4	44.0	43.6	46.8	46.2	50.4	54.4	53.6	57.6	64.2
Kalfpap (C)	**17**	39.4	43.4	45.6	48.0	52.0	56.0	61.8	68.0	72.0	81.8	90.8	103.8
	18	39.2	40.4	43.0	47.6	51.4	54.8	62.8	70.0	75.2	84.8	91.8	106.2
	19	37.4	41.2	41.0	46.2	49.2	50.4	55.4	60.0	64.4	71.2	78.6	90.4
	20	36.0	39.6	42.0	44.4	48.6	52.4	60.0	66.8	73.2	82.6	93.2	107.4
	21	40.0	36.4	42.4	43.8	47.2	48.0	50.8	55.8	65.4	72.8	74.8	87.6
	22	40.8	42.6	42.2	44.6	47.6	52.6	58.6	60.0	65.4	73.4	83.4	87.6
	23	43.2	46.2	46.4	49.4	52.4	55.0	60.4	65.2	70.0	78.6	87.0	94.6
	24	44.2	47.0	49.6	52.2	55.8	60.6	65.2	70.8	79.2	85.8	96.4	102.6
FP-Kalfpap (D)	**25**	43.4	46.6	49.4	53.6	58.8	62.4	67.2	74.2	78.2	88.2	100.4	111.6
	26	38.4	42.4	44.8	42.2	43.6	42.2	41.0	43.6	48.2	47.8	54.0	60.6
	27	39.6	44.0	48.8	46.2	46.2	43.8	43.2	46.4	51.0	53.6	58.6	62.6
	28	39.0	42.4	44.6	42.6	44.4	49.2	58.6	61.8	66.2	72.4	76.8	82.4
	29	37.2	42.4	40.6	43.0	48.8	44.6	45.8	46.8	49.6	53.2	58.0	63.8
	30	44.4	49.2	52.6	53.8	59.0	63.6	67.2	69.8	71.4	86.8	92.8	100.6
	31	48.8	49.0	54.6	55.0	56.6	61.6	67.2	68.6	73.0	79.2	84.6	91.6
	32	43.6	46.2	49.0	49.0	49.2	50.8	56.2	56.8	62.0	68.2	72.6	77.2

Table A.4 The weekly girth circumference measurements (cm) of 32 Holstein bull calves over a 77-day trial with four milk replacer treatments (A, B, C and D).

Treatment and calves		Days											
		0	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**	**70**	**77**
Biomel (A)	**1**	79.0	83.0	86.0	87.0	92.0	95.0	97.0	99.0	103.0	107.0	108.0	112.0
	2	84.0	87.0	87.0	91.0	93.0	97.0	102.0	101.0	107.0	107.0	122.0	113.0
	3	87.0	87.0	89.0	90.0	96.0	99.0	97.0	101.0	105.0	103.0	108.0	108.0
	4	78.0	81.0	86.0	88.0	90.0	93.0	95.0	99.0	102.0	110.0	111.0	105.0
	5	80.0	86.0	88.0	85.0	89.0	90.0	91.0	95.0	102.0	97.0	101.0	109.0
	6	85.0	87.0	89.0	89.0	90.0	93.0	95.0	95.0	96.0	99.0	105.0	104.0
	7	83.0	84.0	85.0	92.0	93.0	95.0	96.0	99.0	101.0	105.0	106.0	108.0
	8	82.0	81.0	83.0	82.0	86.0	88.0	87.0	90.0	93.0	96.0	100.0	104.0
FP-Biomel (B)	**9**	84.0	88.0	93.0	89.0	93.0	90.0	90.0	88.0	94.0	85.0	91.0	91.0
	10	86.0	88.0	97.0	93.0	94.0	95.0	93.0	97.0	96.0	98.0	103.0	105.0
	11	88.0	92.0	93.0	96.0	97.0	95.0	99.0	96.0	98.0	100.0	101.0	102.0
	12	83.0	84.0	85.0	86.0	90.0	90.0	92.0	94.0	100.0	99.0	103.0	110.0
	13	85.0	85.0	86.0	86.0	88.0	87.0	88.0	88.0	91.0	92.0	95.0	93.0
	14	84.0	88.0	89.0	89.0	91.0	91.0	93.0	93.0	95.0	95.0	96.0	94.0
	15	82.0	86.0	87.0	88.0	88.0	85.0	87.0	87.0	88.0	88.0	90.0	89.0
	16	90.0	84.0	83.0	85.0	87.0	87.0	88.0	88.0	89.0	91.0	94.0	93.0
Kalfpap (C)	**17**	80.0	82.0	85.0	82.0	88.0	90.0	93.0	100.0	91.0	100.0	103.0	109.0
	18	81.0	86.0	88.0	90.0	91.0	91.0	98.0	100.0	100.0	109.0	109.0	113.0
	19	76.0	80.0	82.0	84.0	87.0	88.0	87.0	93.0	99.0	96.0	101.0	104.0
	20	80.0	81.0	82.0	84.0	87.0	90.0	94.0	96.0	100.0	102.0	114.0	112.0
	21	83.0	80.0	84.0	84.0	86.0	92.0	92.0	96.0	95.0	98.0	105.0	114.0
	22	83.0	84.0	85.0	86.0	87.0	92.0	93.0	92.0	96.0	98.0	101.0	105.0
	23	82.0	84.0	86.0	87.0	91.0	92.0	97.0	96.0	100.0	104.0	103.0	109.0
	24	86.0	88.0	90.0	91.0	91.0	92.0	98.0	100.0	103.0	105.0	108.0	111.0
FP-Kalfpap (D)	**25**	86.0	88.0	89.0	90.0	93.0	98.0	100.0	100.0	104.0	106.0	111.0	114.0
	26	81.0	84.0	88.0	86.0	86.0	88.0	89.0	87.0	92.0	90.0	99.0	96.0
	27	81.0	85.0	88.0	89.0	88.0	87.0	87.0	88.0	90.0	92.0	99.0	97.0
	28	80.0	84.0	81.0	85.0	84.0	88.0	90.0	91.0	94.0	98.0	101.0	103.0
	29	83.0	84.0	85.0	88.0	89.0	89.0	89.0	90.0	89.0	92.0	99.0	98.0
	30	83.0	88.0	87.0	89.0	94.0	94.0	95.0	97.0	100.0	103.0	107.0	108.0
	31	86.0	86.0	89.0	90.0	90.0	95.0	96.0	98.0	97.0	92.0	99.0	97.0
	32	84.0	88.0	87.0	91.0	90.0	89.0	91.0	91.0	94.0	97.0	100.0	100.0

Table A.4 The weekly cumulative milk replacer dry mater intake (kg) of 32 Holstein bull calves over a 77-day trial with four milk replacer treatments (A, B, C and D).

Treatment and calves		Days								
		7	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**
Biomel (A)	**1**	4.59	5.58	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	2	4.59	5.58	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	3	4.59	5.58	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	4	4.31	5.58	6.30	6.30	6.60	6.30	6.30	6.30	3.90
	5	4.59	5.58	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	6	5.67	6.30	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	7	5.67	6.30	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	8	5.22	5.04	5.34	5.70	6.30	6.30	6.30	6.30	3.90
FP-Biomel (B)	**9**	5.13	6.12	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	10	5.13	6.12	6.30	6.30	6.00	6.00	6.30	6.30	3.90
	11	5.67	6.30	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	12	4.41	4.95	5.31	6.12	6.30	6.30	6.30	6.30	3.90
	13	4.95	6.12	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	14	5.22	6.12	6.30	6.30	6.30	6.15	5.90	6.00	3.90
	15	5.22	6.12	6.30	6.30	6.00	6.30	5.85	6.30	3.90
	16	5.13	6.12	6.30	5.70	5.70	6.30	6.30	6.30	3.90
Kalfpap (C)	**17**	4.59	5.58	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	18	4.59	5.58	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	19	4.59	5.58	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	20	4.59	5.58	6.23	6.30	6.30	6.30	6.30	6.30	3.90
	21	4.68	5.04	5.40	6.30	6.30	6.30	6.30	6.30	3.90
	22	5.40	4.58	5.46	6.30	6.30	6.30	6.30	6.30	3.90
	23	5.22	6.12	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	24	5.22	6.12	6.30	6.30	6.30	6.30	6.30	6.30	3.90
FP-Kalfpap (D)	**25**	4.41	4.95	5.31	6.12	6.30	6.30	6.30	6.30	3.90
	26	5.13	6.12	6.30	6.20	6.30	6.30	6.30	6.30	3.90
	27	5.13	6.12	6.00	5.00	5.40	6.30	6.30	6.30	3.90
	28	5.40	6.30	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	29	5.22	6.12	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	30	5.22	6.12	6.30	6.30	6.30	6.30	6.30	6.30	3.90
	31	5.67	6.12	6.21	6.00	6.30	6.30	6.30	6.30	3.90
	32	5.40	6.30	6.30	6.30	6.30	6.30	6.30	6.30	3.90

Table A.5 The weekly cumulative starter meal dry mater intake (kg) of 32 Holstein bull calves over a 77-day trial with four milk replacer treatments (A, B, C and D).

Treatment and calves		Days										
		7	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**	**70**	**77**
Biomel (A)	**1**	1.714	2.908	3.682	3.316	6.003	6.572	9.746	13.432	18.683	28.964	31.859
	2	1.552	1.697	2.142	3.277	4.500	7.686	9.261	10.553	15.732	26.902	31.473
	3	1.505	1.351	2.190	3.263	4.088	4.841	6.399	8.340	15.505	13.882	25.291
	4	0.268	0.514	2.825	3.684	4.107	4.891	4.897	8.043	11.991	21.001	22.243
	5	0.754	2.011	1.419	1.240	2.640	3.014	3.238	4.857	9.736	16.315	22.385
	6	0.541	0.225	1.234	0.230	0.834	1.583	2.001	3.965	14.035	15.867	20.133
	7	1.015	1.216	1.769	2.283	4.711	5.764	6.236	13.841	10.159	19.995	24.005
	8	0.048	0.084	0.368	1.218	0.659	1.123	0.240	2.213	6.539	15.008	19.658
FP-Biomel (B)	**9**	0.294	0.747	4.974	0.446	0.366	1.944	2.530	0.192	0.122	5.721	7.850
	10	0.930	2.689	0.716	0.685	1.865	3.803	2.535	2.782	6.767	15.990	20.010
	11	0.643	1.357	1.879	1.151	3.007	4.930	6.740	0.864	0.919	3.985	6.015
	12	0.958	1.469	1.407	0.612	3.750	3.912	2.605	7.901	9.863	26.183	27.500
	13	0.088	0.300	1.112	0.988	1.012	0.316	0.684	2.610	3.066	9.835	7.391
	14	0.391	0.067	1.742	2.272	1.028	1.676	1.520	1.780	3.895	11.970	6.526
	15	0.606	0.852	0.142	0.894	0.511	0.147	0.130	0.402	1.974	9.976	4.670
	16	0.110	0.148	0.255	0.789	1.816	1.892	1.319	1.896	2.415	9.993	15.274
Kalfpap (C)	**17**	0.550	1.491	1.071	2.505	3.085	4.788	3.968	8.646	12.034	19.895	26.628
	18	0.762	2.203	3.054	4.102	5.858	7.875	9.665	11.994	15.979	24.101	28.819
	19	0.493	0.495	1.525	2.799	3.598	4.476	6.795	6.375	11.447	18.052	27.867
	20	0.971	2.142	2.426	4.028	6.013	6.785	9.042	11.242	16.020	25.028	31.196
	21	0.169	0.295	1.319	2.324	1.915	4.015	9.212	11.938	16.028	15.304	23.987
	22	1.042	1.704	1.254	1.540	3.904	7.224	9.147	8.015	16.111	26.011	26.003
	23	0.818	1.405	1.197	2.080	1.867	5.133	5.850	6.150	14.203	22.103	23.995
	24	0.682	2.207	2.511	3.100	4.904	7.096	5.794	12.206	15.896	21.976	22.004
FP-Kalfpap (D)	**25**	0.891	1.632	7.756	4.597	5.706	7.410	11.230	9.487	13.714	28.016	37.500
	26	0.910	0.736	0.674	0.722	0.494	0.952	1.332	0.675	0.622	9.733	12.887
	27	0.436	0.356	0.797	0.966	1.518	1.999	2.497	3.400	4.800	11.569	11.829
	28	0.879	2.651	0.895	1.871	3.908	7.988	9.919	5.033	11.850	29.986	26.014
	29	0.035	4.126	2.539	1.800	1.302	0.998	0.260	1.682	3.153	13.899	16.101
	30	0.573	1.906	2.021	3.845	4.155	5.926	3.504	1.936	11.562	15.948	22.052
	31	1.064	1.477	0.858	1.085	5.196	5.167	5.969	7.181	9.139	19.847	23.989
	32	2.076	2.759	1.769	3.236	1.904	5.777	5.500	4.021	11.836	29.986	26.014

Table A.6 The predicted weights (PW) in kg that correlates to Nandrea Health Products girth circumference (GC) measuring tape in cm. This is not the complete tape but include the values used for Chapter 5.

	Predicted weight in kg					
GC (cm)	**70-79**	**80-89**	**90-99**	**100-109**	**110-119**	**120-129**
0		47.0	63.4	83.8	111.6	
1		48.0	65.4	85.6	114.4	
2		49.0	67.6	87.2	117.6	148.6
3		50.0	69.8	90.0	119.6	
4		51.0	71.6	93.2	123.8	
5		53.4	73.6	96.6		
6	43.0	55.4	75.8	99.0		
7		57.2	77.8	102.0		
8	45.0	59.6	79.8	105.6		
9	46.2	61.6	81.8	108.4		

Table A.7 The predicted weights (PW) in kg adjusted from Nandrea Health Products girth circumference (GC) measuring tape in cm with y = 0,9217x-6,7005. Only for the values used for Chapter 5 adjusted from 32 Holstein bull calves and not the complete tape.

	Predicted weight in kg					
GC (cm)	**70-79**	**80-89**	**90-99**	**100-109**	**110-119**	**120-129**
0		36,6	51,7	70,5	96,2	
1		37,5	53,6	72,2	98,7	
2		38,5	55,6	73,7	101,7	130,3
3		39,4	57,6	76,3	103,5	
4		40,3	59,3	79,2	107,4	
5		42,5	61,1	82,3		
6	32,9	44,4	63,2	84,5		
7		46,0	65,0	87,3		
8	34,8	48,2	66,9	90,6		
9	35,9	50,1	68,7	93,2		