Dietary Supplements to Support Dairy Calf Health and Welfare

by

Brooke Katherine McNeil

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Animal Biosciences

Guelph, Ontario, Canada

© Brooke Katherine McNeil, January, 2023
ABSTRACT

DIETARY SUPPLEMENTS TO SUPPORT DAIRY CALF HEALTH AND WELFARE

Brooke Katherine McNeil
University of Guelph, 2023

Advisor(s): Dr. Trevor DeVries

The objective of this dissertation was to investigate if dietary supplements could improve dairy calf health and welfare. The first study was focused on investigating the effects of *Echinacea purpurea* (**EP**) supplementation on markers of immunity, health, feed intake, and growth of dairy calves. Overall, EP supplementation was associated with blood markers indicative of reduced inflammation and stimulated immunity, with minor benefits to health and growth. The second study was focused on how weaning, as well as supplementing calves with tyndallized *Lactobacillus helveticus* (**TLH**), affects behavioral and physiological indicators of dairy calf affective state. Overall, there were changes in the behavior of calves at weaning that were indicative of a more negative affective state. Supplementation of TLH was associated with indicators of a more negative affective state, especially during weaning. In summary, this research provides novel information on the effects of dietary supplements on dairy calf health and welfare.
ACKNOWLEDGEMENTS

I owe a huge thank you to my advisor, Dr. Trevor DeVries. Trevor, thank you so much for giving me the opportunity to join your lab - “Cowcrew”. I loved being a part of Cowcrew and I want to thank you for creating such a positive lab environment. I truly appreciated all of your guidance throughout my Masters. I could always rely on your quick responses and editing, and detailed feedback which was all so helpful to me. Moving out of the Maritimes alone and adjusting to a new and much bigger province for the first time, enrolling in a busy graduate program, and facing Covid was a lot for me to handle all at once. Your patience and understanding for struggles I faced will forever be remembered and appreciated! I also want to thank my advisory committee members, Dr. Michael Steele and Dr. David Renaud. Mike, you always pushed me to expand on the biological mechanisms in my writing, this improved my papers so much and helped me to advance this literature investigation skill. Thank you also for your enthusiasm and encouragement to try new things, it resulted in some really novel components to my research! Dave, your quick editing and feedback was really appreciated and helped me so much in the progression of my work. Your animal health expertise was so valuable to improving the quality of my research and papers. I only wish there were more opportunities to have connected in person! Trevor, Mike, and Dave - I feel so grateful to have had you all as my advisory committee. From your support and guidance, I’ve not only grown as a researcher, but also as a person!

My research would not have been possible without the generous funding and research support from the Ontario Agri-Food Innovation Alliance Research Program of the University of Guelph and the Ontario Ministry of Agriculture, Food and Rural Affairs, Mapleview Agri Ltd., Lallemand, and the Natural Sciences and Engineering Research Council of Canada. Furthermore, the staff at the research facilities involved played a vital role in calf care and the overall success of the projects – thank you to the staff at Mapleview Agri Ltd., Ponsonby General Animal Facility, and the Ontario Dairy Research Center. Past and present Cowcrew lab mates, it has been so great and so much fun to get to know you all! Thanks so much for your support in my work and help with my projects! I have wonderful memories with you all and I hope we keep in touch.

Finally, I have so many amazing friends and family to thank for all of their love and support along the way although there are a few of them who I would like to especially thank. My parents - Jim and Nancy McNeil, thank you for always believing in me and encouraging me to go after what I am passionate about! My best friend, Kelsey Henneberry, you have been with me through thick and thin and always have the best advice, I am eternally grateful for your friendship! Keltie and Nick Elliot, your hard work ethic and dedication to animal well-being and the dairy industry is so inspiring to me, and thank you for always being there for me and cheering me on! Meagan King, and Sarah Parsons, you are the sweetest friends and best role models. I don’t think I would have made it through these past few years in Ontario without you! Thank you for sharing your homes and your pups with me!
# TABLE OF CONTENTS

Abstract ................................................................................................................................. ii  
Acknowledgements ................................................................................................................ iii  
Table of Contents .................................................................................................................... iv  
List of Tables ........................................................................................................................ vii  
List of Figures ........................................................................................................................ ix  
List of Abbreviations ............................................................................................................. xii  

1  CHAPTER 1: GENERAL INTRODUCTION ....................................................................... 1  
   1.1  Dairy Calf Health and Welfare .................................................................................... 1  
      1.1.1  Nutritional Effects on Calf Health and Welfare .................................................. 3  
      1.1.2  Housing and Stressor Effects on Calf Health and Welfare .................................... 5  
   1.2  Dietary Supplements to Support Dairy Calf Health and Welfare ............................... 8  
      1.2.1  *Echinacea purpurea* ......................................................................................... 10  
      1.2.2  *Lactobacillus helveticus* .................................................................................. 13  
   1.3  Objectives and Hypotheses .......................................................................................... 16  

2  CHAPTER 2: EFFECTS OF *ECHINACEA PURPUREA* SUPPLEMENTATION ON  
   MARKERS OF IMMUNITY, HEALTH, INTAKE, AND GROWTH OF DAIRY CALVES... 18  
   2.1  INTRODUCTION ......................................................................................................... 18  
   2.2  MATERIALS AND METHODS .................................................................................... 21  
      2.2.1  Animals and Housing .......................................................................................... 21  
      2.2.2  Feeding and Health Management ........................................................................ 22  
      2.2.3  Treatments Allocation ......................................................................................... 25  
      2.2.4  Sample Size Calculation ...................................................................................... 27
2.2.5 Measurements and Sample Collection ................................................................. 27
2.2.6 Statistical Analyses ................................................................................................. 32
2.3 RESULTS ..................................................................................................................... 34
2.3.1 Blood ......................................................................................................................... 35
2.3.2 Health ......................................................................................................................... 35
2.3.3 Intake and Growth ..................................................................................................... 37
2.4 DISCUSSION ................................................................................................................ 37
2.5 CONCLUSION .............................................................................................................. 49
2.6 ACKNOWLEDGEMENTS ............................................................................................ 49

3  CHAPTER 3: EFFECTS OF WEANING AND TYNDALLIZED LACTOBACILLUS HELVETICUS SUPPLEMENTATION ON DAIRY CALF BEHAVIORAL AND PHYSIOLOGICAL INDICATORS OF AFFECTIVE STATE ........................................................................... 72

3.1 INTRODUCTION ........................................................................................................... 72
3.2 MATERIALS AND METHODS .................................................................................... 77
3.2.1 Animals and Housing ............................................................................................... 77
3.2.2 Feeding and Health Management .......................................................................... 80
3.2.3 Treatments Allocation ............................................................................................. 83
3.2.4 Measurements and Sample Collection ................................................................... 84
3.2.5 Statistical Analyses ................................................................................................. 90
3.3 RESULTS ....................................................................................................................... 92
3.4 DISCUSSION ............................................................................................................... 94
3.5 CONCLUSION .............................................................................................................. 102
3.6 ACKNOWLEDGMENTS ............................................................................................. 103

4  CHAPTER 4: GENERAL DISCUSSION ......................................................................... 114
4.1 Important Findings .................................................................................................... 114
4.2 Limitations and Future Research................................................................. 117

4.3 Implications................................................................................................. 121

References.......................................................................................................... 123
LIST OF TABLES

Table 2.1. Ingredient and chemical composition (mean ± SD) of the calf starter, combo feed¹, calf grower feed¹ and milk replacer fed to all calves ................................................................. 51

Table 2.2. Feeding program for all calves .............................................................................. 52

Table 2.3. Analysis results (mean ± SD) of the Echinacea purpurea samples fed to calves on those treatments that received it ................................................................. 53

Table 2.4. Multivariable linear regression model of the variables associated with blood haptoglobin concentration, segmented neutrophil count, lymphocyte count, and segmented neutrophils/lymphocytes ratio (N:L ratio). ........................................................................... 54

Table 2.5. Medication treatment protocol .............................................................................. 55

Table 2.6. Multivariable linear regression model of the variables associated with blood white blood cell, band neutrophil, monocyte, and basophil counts ........................................................................ 56

Table 2.7. Multivariable linear regression model of the variables associated with the blood IL-10, IL-6, and TNF-α concentrations .......................................................................... 57

Table 2.8. Multivariable linear regression model of the variables associated with the proportion of fecal scores that were abnormal (scores 3 or 4; abnormal FS), proportion of fecal scores that were severe (score 4; severe FS), electrolyte doses, and the proportion of respiratory scores ≥ 4 (RS ≥ 4) in the milk-fed period .............................................................................. 58

Table 2.9. Cox proportional hazards model evaluating treatment for diarrhea with: 1) Oral Metacam (Meloxicam; Diarrhea Treatment 1) and with 2) Oral Metacam (Meloxicam) and Borgal (Trimethoprim and Sulfadoxine; Diarrhea Treatment 2) ........................................................................... 59

Table 2.10. Multivariable logistic regression model of the variables associated with the risk of bovine respiratory disease (BRD) and the risk of mortality in the milk-fed period .................... 60
Table 2.11. Multivariable linear regression model of the variables associated with milk replacer (MR) intake, grain intake, and feed conversion rate (FCR) in the milk-fed period. .......................... 61

Table 2.12. Multivariable linear regression model of the variables associated with calf hip height, weekly BW, and average daily gain (ADG) in the milk-fed period. ................................. 62

Table 2.13. Multivariable linear regression model of the variables associated with the proportion of respiratory scores with no symptoms of bovine respiratory disease (RS = 0), grain intake, average daily gain (ADG), and feed conversion rate (FCR) in the post-wean period ......................... 63

Table 2.14. Multivariable logistic regression model of the variables associated with the risk of bovine respiratory disease (BRD) and the risk of mortality in the post-wean period ..................... 64

Table 2.15. Cox proportional hazards model evaluating treatment for respiratory disease with Nuflor (Florfenicol) and Metacam SQ (Meloxicam; Respiratory Treatment 1), including the milk-fed and post-wean periods ................................................................. 65

Table 3.1. Ingredient and chemical composition (mean ± SD) of the calf starter and milk replacer fed to all calves. ........................................................................................................ 104

Table 3.2. Description of recorded play behavior. adapted from that of previous studies (Jensen et al., 1998; Mintline et al., 2013) ........................................................................................................ 105
LIST OF FIGURES

Figure 2.1. Eosinophils by treatment and arrival BW. Each dot represents a calf. The treatments included: CON = control (calves received no *Echinacea purpurea*), E14 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28, and E56 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56. N=39 calves/treatment. ............ 66

Figure 2.2. Rectal temperature (back-transformed mean +/- 95% CI) by d (a = 14, b = 28, and c = 57), treatment and FTPI status. FTPI = failed transfer of passive immunity, defined as STP values < 5.2 g/dL. Within FTPI status (no or yes), significant differences (P ≤ 0.05) detected between treatments are denoted by ‘*’, and tendencies for differences (0.05 < P ≤ 0.1) are denoted by ‘†’. The treatments included: CON = control (calves received no *Echinacea purpurea*), E14 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28, and E56 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56. N=39 calves/treatment. .................................................................................................................. 67

Figure 2.3. Proportion (mean +/- SE) of respiratory scores with no symptoms of bovine respiratory disease (RS = 0) in the milk-fed period by treatment and source. Within each source, significant differences (P ≤ 0.05) detected between treatments are denoted by ‘*’, and tendencies for differences (0.05 < P ≤ 0.1) are denoted by ‘†’. The treatments included: CON = control (calves received no *Echinacea purpurea*), E14 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28, and E56 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56. N=80 calves/treatment. ......................................................................................... 68

Figure 2.4. Proportion (back-transformed mean +/- 95% CI) of respiratory scores ≥ 4 (RS ≥ 4) in the post-wean period by treatment and source. Within each source, significant differences (P ≤ 0.05) detected between treatments are denoted by ‘*’, and tendencies for differences (0.05 < P ≤ 0.1) are denoted by ‘†’. The treatments included: CON = control (calves received no *Echinacea purpurea*), E14 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28, and E56 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56. N=80 calves/treatment. ........................................................................................................ 69
Figure 2.5. Average post-wean period weekly body weight by treatment and arrival body weight. Each dot represents a calf. The treatments included: CON = control (calves received no *Echinacea purpurea*), E14 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28, and E56 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56. N=80 calves/treatment........................................................................................................... 70

Figure 2.6. Diagram of the room: milk-fed period layout on the left, individual stalls were 101.6 cm tall (A), 78.74 cm wide (B), 121.92 cm long (C), and the post-weaning period pens (pods) on the right were the same height, 198.12 cm wide (D) and 396.24 cm long (E). ........................................... 71

Figure 3.1. Cognitive test (detour task) pen layout. A = pen entrance, B = detour apparatus... 106

Figure 3.2. Example of a calf eye infrared thermography image used to determine maximum eye temperature. The red triangle placement is where the software program (FLIR Tools) detected is the maximum temperature within the circle placed around the calf’s eye (lacrimal caruncle). . 107

Figure 3.3. The number of lying bouts (count/d; a), the average lying bout length (average min/bout; b), and the time spent lying (min/d; c) by treatment and day (mean ± SE). The treatments include: CON = control (calves received no *Lactobacillus helveticus*; n = 11) and TLH = calves received 5 g/d of tyndallized *Lactobacillus helveticus* at 10⁹ CFU/g, split over 2 milk feedings from d 3-42 (n = 12). The vertical dashed line indicates when weaning began (d 35) .................................................................................................................................................. 108

Figure 3.4. Total play duration (s/d; a), and total play count (count/d; b) by treatment and d (mean ± SE). The total play duration was determined by the total time the calf spent running and/or rubbing shavings, while the total play count was determined by the total count of bucks, kicks, jumps, head-shake/swings, and head-butts the calf performed (all behaviors defined in Table 2) during a 210 second play assessment conducted around the time of bedding (0900 h). The treatments include: CON = control (calves received no *Lactobacillus helveticus*; n = 11) and TLH = calves received 5 g/d of tyndallized *Lactobacillus helveticus* at 10⁹ CFU/g, split over 2 milk feedings from d 3-42 (n = 12). The vertical dashed line indicates when weaning began (d 35) .................................................................................................................................................. 109
Figure 3.5. Cognitive test duration (mean ± SE) by treatment and d. The treatments include: CON = control (calves received no *Lactobacillus helveticus*; n = 11) and TLH = calves received 5 g/d of tyndallized *Lactobacillus helveticus* at $10^9$ CFU/g, split over 2 milk feedings from d 3-42 (n = 12).

Figure 3.6. Saliva cortisol (mean ± SE) by treatment and d. The treatments include: CON = control (calves received no *Lactobacillus helveticus*; n = 11) and TLH = calves received 5 g/d of tyndallized *Lactobacillus helveticus* at $10^9$ CFU/g, split over 2 milk feedings from d 3-42 (n = 12). The vertical dashed line indicates when weaning began (d 35).

Figure 3.7. Maximum eye temperature (mean ± SE) by treatment and d. The treatments include: CON = control (calves received no *Lactobacillus helveticus*; n = 11) and TLH = calves received 5 g/d of tyndallized *Lactobacillus helveticus* at $10^9$ CFU/g, split over 2 milk feedings from d 3-42 (n = 12). The vertical dashed line indicates when weaning began (d 35).

Figure 3.8. Blood serotonin (mean ± SE) by treatment and d. The treatments include: CON = control (calves received no *Lactobacillus helveticus*; n = 11) and TLH = calves received 5 g/d of tyndallized *Lactobacillus helveticus* at $10^9$ CFU/g, split over 2 milk feedings from d 3-42 (n = 12). The vertical dashed line indicates when weaning began (d 35).
LIST OF ABBREVIATIONS

NCD – neonatal calf diarrhea
BRD – bovine respiratory disease
MR – milk replacer
CON – control treatment
CP – crude protein
NDF – neutral detergent fiber
ADF – acid detergent fiber
DM – dry matter
SD – standard deviation
CV – coefficient of variation
SE – standard error

Chapter specific terminology

Chapter 2:

EP – Echinacea purpurea

E14 – treatment where calves received 3 g/d of powdered EP extract split over 2 milk feedings from d 14 to 28

E56 – treatment where calves receiving 3 g/d of EP split over 2 milk feedings from d 1 to 56

BW – body weight
Pod – a group of 5 consecutively housed calves receiving the same treatment

WBC – white blood cell

ADG – average daily gain
STP – serum total protein
FTPI – failed transfer of passive immunity
DMI – dry matter intake
NRC – National Research Council
ME – metabolizable energy
FCR – feed conversion rate
N:L ratio – segmented neutrophil: lymphocyte ratio
FS – fecal score
RS = 0 – the proportion of respiratory scores with no symptoms of bovine respiratory disease
RS ≥ 4 – the proportion of respiratory scores ≥ 4

Chapter 3:

LH – *Lactobacillus helveticus*

TLH – tyndallized *Lactobacillus helveticus*, and represents the treatment where calves received 5 g/d of tyndallized *Lactobacillus helveticus* at 10⁹ CFU/g

CR – colostrum replacer

MET – maximum eye temperature
1 CHAPTER 1: GENERAL INTRODUCTION

Calf health and welfare plays a major role in farm profitability (Asheim et al., 2016), public perception (Ly et al., 2021), and therefore, industry sustainability. Furthermore, healthy calves may support farmer mental health, as associations between dairy cow health and welfare and farmer mental health have been detected (King et al., 2021).

This review includes an overview of dairy calf disease prevalence, the primary diseases that impact dairy calves and pathogens involved, and consequences of disease. Next, animal welfare assessment is outlined. Then, nutritional, housing, and stress-related management factors involved in dairy calf health and welfare are summarized. An overview is given on one management practice that is an emerging area in animal agriculture and the focus of this thesis - dietary supplements - in relation to dairy calf health and welfare. Specifically, an overview is provided of *Echinacea purpurea* and tyndallized *Lactobacillus helveticus*, the specific dietary supplements investigated in this thesis. The information provided on these supplements include background information, their use in the literature to date, and proposed mechanisms of action.

1.1 Dairy Calf Health and Welfare

Dairy calf morbidity is a global issue. The overall calf morbidity rate is reported as 33.9% in the United States (n = 2,545 dairy calves; Urie et al., 2018). The main illnesses that affect dairy calves include neonatal calf diarrhea (*NCD*) and bovine respiratory disease (*BRD*; Windeyer et al., 2014). Windeyer et al. (2014) reported more than 23% and almost 22% of calves
were treated for NCD and BRD, respectively (n = 2,874 calves on dairy farms in Minnesota, United States, and Ontario, Canada). Similarly, Medrano-Galarza et al. (2018) reported that 23% and 17% of calves were diagnosed with NCD and BRD, respectively (n = 1,488 calves on dairy farms in Ontario, Canada). Diarrhea most commonly occurs in calves less than 30 days of age, while BRD more commonly affects calves greater than 30 days of age (Svensson et al., 2006; Urie et al., 2018). Various pathogens have been reported to be involved in the development of NCD and BRD including bacteria, viruses, and protozoa, and often, co-infection rather than just one pathogen is to blame (Bowland and Shewen, 2000; Cho and Yoon, 2014). Common pathogens that cause diarrhea in dairy calves include Escherichia coli, Salmonella spp, Clostridium perfringens, bovine rotavirus, bovine coronavirus, bovine viral diarrhea virus, and Cryptosporidium parvum (Constable et al., 2004; Todd et al., 2010; Cho and Yoon, 2014). While primary pathogens involved in BRD include bovine coronavirus, bovine respiratory syncytial virus, Mannheimia haemolytica, Pasteurella multocida, Histophilus somni, and Mycoplasma spp. (Griffin et al., 2010; Francoz et al., 2015; Doyle et al., 2017; Yaman et al., 2018; Snyder and Credille, 2020). There are many consequences of disease including increased risk of mortality (Windeyer et al., 2014; Urie et al., 2018), reduced long term productivity (Schaffer et al., 2016), and increased expenses and consequently economic loss for the producer through treatment and impacts on health and production (Ayrle et al., 2016; Delabouglise et al., 2017).

The primary components of animal welfare assessment include basic health and functioning, affective states, and natural living (Fraser, 2008). The affective state of an animal is
how they feel; affective states can be positive such as pleasure, happiness, and contentment, or negative such as pain, frustration, and fear (Ede et al., 2019). Natural living includes an animal being able to perform behaviors that are believed to be important to the species (Fraser et al., 1997). Evidently, disease not only negatively affects dairy calf health, but also their welfare. Although, there are many recommended management practices to reduce disease/support health and development, promote more positive affective states, and allow for natural behaviors.

1.1.1 Nutritional Effects on Calf Health and Welfare

Nutritional approaches are directly involved in supporting dairy calf health and welfare. Firstly, colostrum, the first milk produced by a cow after giving birth, contains high levels of fat and protein for the neonatal calf, immunoglobulins for passive immunity, and a number of other beneficial bioactive components (Hammon et al., 2013; Lopez and Heinrichs, 2022). Colostrum is vital to immunity and survival in calves (Godden et al., 2019). The calf must consume a sufficient amount (3-4 L for the average Holstein calf) of clean, high-quality (adequate amount of immunoglobulins) colostrum as soon as possible (ideally 1-2 h) after birth to acquire immunity from transfer of the colostral immunoglobins (Godden et al., 2019). The immunoglobulins acquired from colostrum provide the calf with immunity until their own immune system begins to develop at a few weeks of age (Hulbert and Moisa, 2016). Conversely, inadequate colostrum consumption, which results in failed transfer of passive immunity, is associated with increased risk of disease (Windeyer et al., 2014). Transition milk, defined as milkings 2-6 from the cow after birth (Godden, 2008), contains less immunoglobulins than
colostrum but more than whole milk (Hare et al., 2020). Researchers have reported health
benefits of extended colostrum or transition milk feeding programs compared to an early and
direct switch to whole milk/milk replacer (Kargar et al., 2020; Van Soest et al., 2022).

The daily volume of milk fed to pre-weaned calves varies among farms (Medrano-
Galarza et al., 2017); however, there is research that supports offering higher volumes of milk
due to improved health (Jorgensen et al., 2017), growth (Khan et al., 2011), and welfare
(Rosenberger et al., 2017; Palczynski et al., 2020) outcomes. High planes of milk nutrition are
especially important for dairy calves raised outdoors in the winters, as increased dietary energy
can counteract energy loss from cold weather (Jaster et al., 1990). Delaying weaning calves off
milk, for example, from 6 to 8 wk of age, can also have welfare benefits, as evidenced by less
behavioral indicators of stress (Eckert et al., 2015). Later weaning also better aligns with what
would occur if the calf were to be raised by their dam in a natural environment (Reinhardt and
Reinhardt, 1981). Furthermore, step down weaning methods are superior to abrupt weaning
especially in terms of supporting gastrointestinal and overall health and functioning (Steele et al.,
2017) and welfare (Enriquez et al., 2011).

Consumption of solid feed by dairy calves alters the composition of rumen microbes and
contributes to rumen development, which is required to digest larger quantities of solid feed
post-weaning (Khan et al., 2016). Therefore, it is important that calves are consuming solid feed
prior to weaning, as if they are not, it is likely that post-weaning they will not be able to adapt to
consuming solid feed quick enough to meet their daily nutrient and energy requirements
(Benetton et al., 2019). In fact, some weaning methods are based on solid feed consumption on an individual calf basis, whereby the calf only begins weaning depending on their consumption of solid feed (Roth et al., 2009). Furthermore, provision of chopped hay or straw can benefit gastrointestinal tract development (Khan et al., 2016; Chen et al., 2021; Gasiorek et al., 2022) and result in behaviors indicative of improved welfare compared to calves not offered it (Mattiello et al., 2002; Horvath and Miller-Cushon, 2019).

### 1.1.2 Housing and Stressor Effects on Calf Health and Welfare

In addition to nutrition and its management, there are many other factors that influence dairy calf health and welfare and, therefore, require careful management. Calf behavior, in addition to housing environment, often facilitate pathogens to be easily picked up and spread due to indiscriminate defecation and fecal-oral method of pathogen transfer (Barrington et al., 2002) and poor ventilation resulting in high bacteria counts in the air (Gorden and Plummer, 2010). Provision of sufficient, clean, and dry bedding reduces the risk of disease and improves calf comfort (Lago et al., 2006; Ninomiya and Sato, 2009; Camiloti et al., 2012). Increasing the amount of clean bedding and/or the addition of straw bedding can provide thermal insulation to calves in cold weather further improving comfort and reducing the risk of pneumonia (Nikkhah and Alimirzaei, 2022). To minimize disease transfer between calves, regular pen cleaning to remove soiled bedding and prevent build up of a deep wet bedding pack, pen disinfection between calves, increased space/calf and avoiding overstocking is recommended (Lago et al., 2006; McGuirk et al., 2008; Medrano-Galarza et al., 2018). As well, exposure to weaned/older
cattle has been identified as a risk factor for disease in pre-weaned dairy calves (Medrano-Galarza et al., 2018). Sufficient ventilation and monitoring humidity levels are important for air quality and supporting respiratory health (Lago et al., 2006; Urie et al., 2018). Group housing supports the “natural living” and “affective states” components of animal welfare assessment, as cattle are herd animals (Stull and Reynolds, 2008). Although some researchers have reported increased risk of disease in group housing dairy calves (Buczinski et al., 2018), group housing is associated with improved cognition evidenced through increased feed intake (Costa et al., 2015) and behaviors indicative of reduced stress in dairy calves (Veissier et al., 1997; compared to individual housing), and is therefore recommended by researchers (Costa et al., 2016).

There are some common practices that often cause stress to dairy calves, which can reduce immunity (Hulbert and Moisa, 2016) and negatively effect calf health. Weaning is an experience all dairy calves go through; there are many reasons why it may impact dairy calf affective state. Milk feeding, which is often delivered through a nipple, facilitates sucking behavior which allows performance of a motivated behavior and may elicit satiety and calming affects as previously cited; weaning removes these experiences that may contribute to a positive affective state. Calves prefer to drink milk (Webb et al., 2014) and it is their natural source of nutrition in early in life (Khan et al., 2016); therefore, removal of milk could inflict negative affect, especially when early weaning is practiced. Additionally, there is high variability among calves regarding when they begin to eat solid feed and how quickly their consumption increases during weaning (de Passille and Rushen, 2016). Yet abrupt weaning methods still occur (Vasseur
et al., 2010), which can result in growth depressions (Steele et al., 2017). In addition, on most dairy farms in North America, calves are weaned much earlier (6-8 wk of age; Vasseur et al., 2010) than they would be if raised by their dam in a natural environment (average 10 mo; Reinhardt and Reinhardt, 1981). Calves weaned early show more signs of hunger (negative affect) than calves weaned later and/or by a specified amount of voluntary starter intake (hunger inferred by more unrewarded visits to an automated milk feeder; de Passille and Rushen, 2016).

In addition to weaning, there are some other notable common dairy calf stressors. Many calves, especially male calves, who often leave their original farm to be raised at a grower facility, experience transportation (Wilson et al., 2020). Research supports physiological and behavioral changes that support transportation is stressful to dairy calves; for many reasons including handling, the experience, and novelty (environment, other animals, etc.; Trunkfield and Broom, 1990). Painful procedures such as disbudding and dehorning without proper pain control is another major factor affecting dairy calf welfare (Stafford and Mellor, 2011). Disbudding and dehorning are common procedures on dairy farms (Misch et al., 2007). The use of anesthesia and analgesia have increased over the years, in Ontario, Canada, attributed to calf welfare and public perception concerns as well as increases in awareness, availability, and requirements (Winder et al., 2016).

Overall, nutritional approaches, as well as housing and stress management to support and enhance immunity and minimize exposure to pathogens are important to minimize disease in dairy calves. These are valuable calf care measures to take considering dairy calves are already
vulnerable to developing disease because of their naïve immune system (Cortese, 2009), and having an immature, and still developing gut microbiota population (Malmuthuge and Guan, 2017) – which are involved in immunity and overall animal health (Gomez et al., 2019). Therefore, methods to reduce disease in dairy calves warrant research, and dietary supplements have potential.

1.2 Dietary Supplements to Support Dairy Calf Health and Welfare

Dietary supplements (feed additives) are a growing area in animal agriculture as they have the potential to support and improve animal health and welfare. Antimicrobials are commonly administered to treat disease in livestock; therefore, reduced disease can result in reduced usage of antimicrobials and thus minimize antimicrobial resistance (Ayrle et al., 2016; Urie et al., 2018). Furthermore, prevention of disease is always superior to treatment due to the previously discussed consequences of disease. Supplements that can be administered through the diet, such as through milk or with calf starter, are easily implementable on farm, which is an important consideration for new management practices due to limited time and labor being a common challenge on dairy operations (Charlton and Kostandini, 2020). Some common types of supplements researched in dairy calves include medicinal plants (usually in the form of essential oils or extracts), pre-biotics, and microbial-based supplements including probiotics and postbiotics (Seifzadeh et al., 2017; Zamojska et al., 2021). In many studies, multiple supplements have been administered with the aim of additive beneficial effects, although this does inhibit researchers’ ability to identify direct effects of each individual supplement.
Some common plants with medicinal properties that have been investigated in dairy calves include green tea (Ishihara et al., 2001; Heisler et al., 2020; Reis et al., 2022), oregano (Kolling et al., 2016; Katsoulos et al., 2017; Heisler et al., 2020), thyme (Akbarian-Tefaghi et al., 2018; Wafa et al., 2022), garlic (Ghosh et al., 2011; Kekana et al., 2020), eucalyptus (Seifzadeh et al., 2017; Akbarian-Tefaghi et al., 2018), and echinacea (Seckin et al., 2018; Ayrle et al., 2021). Although dependent on the specific plant, the general mode of action is eliciting immunomodulatory, anti-inflammatory, antioxidant, and/or antimicrobial effects through their phytochemicals (Giannenas et al., 2013; Granados-Chinchilla, 2017).

Prebiotics are non-digestible food ingredients that stimulate and are utilized by beneficial microorganisms (Singla and Chakkaravarthi, 2017). To date, the most common prebiotics supplemented to dairy calves are carbohydrates, specifically oligosaccharides (Uyeno et al., 2015). Prebiotics can enhance the benefits of microbial supplements, including probiotics, and are therefore often supplemented together (Cangiano et al., 2020).

Probiotics are beneficial live microorganisms that improve health when consumed in an adequate amount (FAO and WHO, 2002). Whereas, postbiotics are dead probiotics, most often from heat-killing (also referred to as “tyndallized probiotics”; Adams, 2010; Piqué et al., 2019), made up of non-viable microbial cells and metabolites (Noh et al., 2022). Probiotics have received most of the focus in the literature to date, regarding effects of microbial-based supplements on gut, general health, and mental well-being. Although, postbiotics have gained attention since they can yield similar beneficial affects as probiotics while being safer, in that
they cannot cause infection, and are easier to transport and store (Adams, 2010; Piqué et al., 2019). More research is needed on probiotics, and especially on postbiotics, as the modes of action of these are not well understood (Deshpande et al., 2018; Piqué et al., 2019; Cuevas-González et al., 2020). Although, some general proposed mechanisms of both probiotics and postbiotics include improving the gut microbial composition (Sanchez et al., 2017; Canani et al., 2017), antagonizing pathogens (Corr et al., 2009; Liu et al., 2017), immunomodulatory and anti-inflammatory effects (Yahfoufi et al., 2018; Taverniti and Guglielmetti, 2011), and modulating levels of neurotransmitters (Lyte and Cryan, 2014; Hara et al., 2018). A wide range of probiotic species (fed as is or converted to postbiotics) have been researched in dairy calves, although in particular, Lactobacillus spp. and Bacillus spp. have been most commonly studied (Uyeno et al., 2015). Of all of these supplements, the medicinal plant, *Echinacea purpurea*, and the postbiotic, tyndallized *Lactobacillus helveticus*, were investigated in this thesis.

### 1.2.1 *Echinacea purpurea*

Echinacea, also known as ‘purple coneflower’, is a herbaceous perennial plant, native to North America, in the Asteraceae family and one of the most well-recognized medicinal plants (Barnes et al., 2005; Kumar and Ramaiah, 2011; Manayi et al., 2015). There are nine species of Echinacea, although *Echinacea purpurea* (EP), *Echinacea angustifolia*, and *Echinacea pallida* are the three used medicinally, with EP the most common (Barnes et al., 2005). Echinacea purpurea has long been used by indigenous peoples for a variety of ailments, although most research in humans is focused on its potential ability to prevent and treat respiratory illness
(Aucoin et al., 2020). Studies supporting such benefits have led to EP becoming a top-selling medical herb (Kumar and Ramaiah, 2011). Although, there are mixed findings among studies on its efficiency, with some researchers reporting EP supplementation was associated with benefits (Weber et al., 2005), and others reporting no detected benefits (Taylor et al., 2003) in terms of improving respiratory illness symptoms in humans. Additionally, the exact modes of action are still not completely understood (Manayi et al., 2015).

While many studies exist on EP supplementation in various livestock species, most studies have been focused on its effects on inflammatory- and immunity-related blood markers and performance outcomes (intake and growth), with little reported on health outcomes. The results of the most commonly reported parameters (e.g., white blood cells, cytokines, and growth measures) are variable among studies, although when considering the results from all studies together, it appears EP supplementation may be beneficial for animals. Importantly, in dairy calves, the effects of EP supplementation remain under-researched. Among the studies that have been conducted in dairy calves, Ismael et al. (2009) who reported that co-administering Echinacea with foot and mouth disease vaccines increased the vaccine’s efficiency in calves. Seckin et al. (2018) reported that supplementation of EP and Pelargonium sidoides to calves elicited an immune response, evidenced through increased IgG and γ-interferon levels and upregulation of γ-interferon IL-1-β, IL-2 and TNF-α. Finally, Ayrle et al. (2021) suggested that EP may activate the local enteric immune system, however, those researchers detected mixed results with EP supplementation in calves.
The active components in EP include: 1) phenolics which exhibit antioxidant effects (Dalby-Brown et al., 2005), 2) alkamides which have anti-inflammatory effects (Chen et al., 2005), and 3) polysaccharides which have immune stimulating abilities (Steinmuller et al., 1993). There is also more recent research on bacterial endophytes in EP and their ability to enhance the immune system, accounting for the majority of the immunomodulation observed (Pugh et al., 2013; Todd et al., 2015; Haron et al., 2016). Specifically, lipopolysaccharides and Braun-type lipoproteins derived from the bacterial endophytes in EP stimulate the body’s macrophages (Tamta et al., 2008; Pugh et al., 2013), which promotes the production of cytokines (both pro-inflammatory, such as TNF-α and IL-6, and anti-inflammatory, such as IL-10; Burger et al., 1997; Sullivan et al., 2008). The effect of bacterial endophytes is supported by research that demonstrated EP grown in a sterile environment lacked both lipopolysaccharides and macrophage stimulating ability (Todd et al., 2015). Furthermore, the addition of substances that degrade bacterial lipopolysaccharides and lipoproteins (polymyxin B and lipoprotein lipase) resulted in diminished immunomodulation activity (Tamta et al., 2008). *Echinacea purpurea* is also reported to increase circulating white blood cells (Goel et al., 2005). Therefore, EP can affect the innate immune system (Haron et al., 2016). Considering adaptive immunity, immunoglobulins have also been investigated, although there are conflicting results: IgG and IgM levels were reported to increase (Rehman et al., 1999) and decrease (Mishima et al., 2004) in response to Echinacea.
There are few studies available in the literature on the effects of EP supplementation on dairy calves, as biological outcomes can vary among animal species, more of such research is needed. Additionally, in most of those studies EP was assessed in combination with other supplements; to identify direct effects of EP, it must be supplemented alone. Furthermore, of the EP supplementation calf studies available, the immune markers assessed are variable, limiting comparisons between studies. Therefore, careful selection and assessment of inflammatory and immune markers commonly reported to be affected by EP supplementation and commonly reported in dairy calf research is needed. Finally, health, intake, and growth outcomes reported in the EP supplementation calf studies available are limited, yet important factors that need to be assessed to be able to make a thoughtful recommendation on if EP supplementation to calves is advisable on commercial farms.

1.2.2 Lactobacillus helveticus

*Lactobacillus helveticus* (LH) is a probiotic that was originally isolated from a dairy culture (Foster et al., 2011). It has been mostly researched in its live form, as a probiotic, while less researched in its dead form, as a postbiotic. This is likely because postbiotics are only recently gaining attention due to increased awareness of their benefits over probiotics including being safer, and easier to transport and store (Piqué et al., 2019). Although no research has been conducted in livestock species, including dairy calves, the supplementation of LH in either form has been found to be beneficial to the mental well-being in rodents and humans. Specifically, considering probiotic research, Liang et al. (2015) reported supplementation of live LH NS8 to
stressed rats lead to reduced behavioral dysfunction (anxiety, depression and cognitive) compared with an anti-depressant (selective serotonin reuptake inhibitor- citalopram). Also, LH was associated with lower plasma corticosterone and adrenocorticotropic hormone levels, higher plasma interleukin-10 levels, restored hippocampal serotonin and norepinephrine levels, and more hippocampal brain-derived neurotrophic factor mRNA expression. Likewise, LH R0052 supplementation was associated with reduced anxiety-like behavior in mice (Ohland et al., 2013), and LH (strain unspecified) improved learning and memory in mice (Ohsawa et al., 2015). Supplementation of LH R0052, in addition to Bifidobacterium longum R0175, was associated with reduced anxiety-like behavior in rats and psychological distress in humans (Messaoudi et al., 2011). While considering postbiotic research, Maehata et al. (2019) reported supplementation of heat-killed (tyndallized) LH (TLH) MCC1848 to stressed mice improved anxiety- or depressive-like behaviors and stress-induced gene expression alterations. Importantly, caution should be used when comparing the results of the above cited literature, as different strains were used, and the effects and mechanisms of probiotics and postbiotics can vary between genres, species, and/or strains (de Almada et al., 2016; Cuevas-González et al., 2020).

The mode of action of probiotics and postbiotics effects on affective state may involve the gut-brain axis, which is the bi-directional communication pathway between the gut and the brain, involving the nervous, endocrine, and immune systems (Makris et al., 2021), which links emotional and cognitive brain regions with gut function (Jenkins et al., 2016). The gut
microbiota plays a key role in this axis whereby undesirable alterations in gut microbiota composition and/or diversity, termed “dysbiosis” and psychological stress are linked (Cryan and Dinan, 2012). Probiotics and postbiotics can modulate the gut microbiota composition and the gut-brain axis (Zhang et al., 2020; Chudzik et al., 2021), positively affecting brain physiology and behavior (Cryan and Dinan, 2012). The exact modes of action and such differences between probiotics and postbiotics are yet to be elucidated (Deshpande et al., 2018; Cuevas-González et al., 2020). Maehata et al. (2019) was the only study identified in the literature that examined the effects of TLH supplementation on the affective state of animals (mice). While those researchers did not detect any effect of TLH supplementation on the gut microbiota composition, they suggested the beneficial effects could be attributable to vagus nerve stimulation, altering the metabolites produced by the gut microbiota, and/or modulating the immune system (cytokines). The vagus nerve extends from the enteric nervous system to the central nervous system, transferring information between the gut and the brain; stimulation of this nerve exerts anti-inflammatory effects and has been used in the treatment of depression (Bonaz et al., 2018). Related, the immune system is involved in mental well-being; humans suffering from depression have higher levels of pro-inflammatory cytokines (Raison et al., 2006). In support of LH having immunomodulatory effects, a live strain (LH SBT2171) of LH was reported to inhibit production of proinflammatory cytokines IL-6 and IL-1β (Yamashita et al., 2014).

Considering microbial-based supplements in general, there is little research available on how probiotics impact affective state in cattle (Kelsey et al., 2018); however, of the studies that
considered this, most reported results that may be indicative of reduced negative affect (Zhang et al., 2016; Kelsey et al., 2018; Lee et al., 2019; Xie et al., 2020). Unfortunately, no studies were identified in the literature that investigated the effects of postbiotics on cattle affective state.

Evidently, more research is needed to investigate the effects of supplemental microorganisms on the affective state of dairy calves. In particular, investigation on the effects of LH (supplemented alone), on affective state of dairy calves is required. Due to the safety and storage benefits of postbiotics compared to probiotics, LH especially as a postbiotic warrants research. Furthermore, physiological and behavioral data is required, as Maehata et al. (2019) did in mice, to consider together, in order to make the most accurate inferences about calf affective state.

1.3 Objectives and Hypotheses

Dairy calves require careful management of nutrition, housing, and stress to prevent or at least minimize disease while maximizing good health and welfare. The investigation of dietary supplements is a growing area in dairy calf research that deserves continued attention due to their potential to reduce antimicrobial use and improve dairy calf health and welfare. Furthermore, as previously explained, there are research gaps in EP and TLH supplementation to dairy calves. Thus, the overall objective of this thesis was to investigate if dietary supplements (specifically EP and TLH) could improve dairy calf health and welfare. This objective was carried out through 2 independent experimental studies. The objective of the first study (Chapter 2) was to
determine the effects of EP supplementation on markers of immunity, health, feed intake, and
growth of dairy calves. The hypothesis was that EP supplementation, especially to calves during
the whole milk-fed period, would reduce inflammation (decreased haptoglobin concentration)
and immune system stimulation (increased cytokine and white blood cell counts), and
consequently, improve the immunity, health, intake, and growth of calves compared to calves not
receiving EP. The objectives of the second study (Chapter 3) were to determine if weaning
would induce physiological and behavioral indicators of negative affective state and if
supplementation of TLH to dairy calves would result in reduced indicators of negative affect
during weaning. The hypothesis was that weaning would induce behavioral and physiological
indicators of negative affective state, and that those indicators would be reduced in calves
receiving TLH.
CHAPTER 2: EFFECTS OF ECHINACEA PURPUREA SUPPLEMENTATION ON MARKERS OF IMMUNITY, HEALTH, INTAKE, AND GROWTH OF DAIRY CALVES

2.1 INTRODUCTION

Statistics on pre-weaned dairy calf morbidity and mortality remain undesirable. A recent estimate of mortality rates of female dairy calves in Canada was 6.4% (n = 1373 farms; Winder et al., 2018), while it was similarly 6.9% in the US considering female and male dairy calves (n = 105 farms; Walker et al., 2012). Several researchers have documented high levels of morbidity, with 33.9% of female dairy calves being treated for disease in Urie et al. (2018; n = 2,545 calves) and 23% and 22% of calves were treated for diarrhea and respiratory disease, respectively, in Windeyer et al. (2014; n = 2,874). Male dairy calves also have a high-level of mortality and morbidity, with 7.5% dying and 88.4% being treated for a health disorder at a veal facility (n = 992 calves; Goetz et al., 2021). These high levels of disease result in high antimicrobial use. Walker et al. (2012) reported 73% and 82% of calves that experienced diarrhea and respiratory disease received an antimicrobial, respectively. Likewise, Urie et al. (2018) reported nearly three-quarters (73.8%) of sick calves were administered an antimicrobial. High antimicrobial use is a concern for many reasons – especially the growing challenge of antimicrobial resistance (Berge et al., 2006). To address these levels of morbidity and mortality evaluating colostrum management, housing design and hygiene, nutrition, pathogen exposure, and stressors including
transportation (Hulbert and Moisá, 2016; Seckin et al., 2018) can aid in the prevention of disease.

Just as in human medicine, natural supplements may be a useful intervention to support and enhance dairy calf immunity and health, potentially serving as a complementary treatment option or alternative to antimicrobials (Seckin et al., 2018). Echinacea is a herbaceous perennial plant indigenous to North America (Barnes et al., 2005), commonly called ‘purple coneflower’ (Kumar and Ramaiah, 2011). There are nine species of Echinacea, although *Echinacea purpurea* (EP), *Echinacea angustifolia*, and *Echinacea pallida* are the 3 used medicinally, with EP the most common (Barnes et al., 2005). Active components in EP include: 1) phenolics which exhibit antioxidant effects (Dalby-Brown et al., 2005), 2) alkamides which have anti-inflammatory effects (Chen et al., 2005), and 3) polysaccharides which have immune stimulating abilities (Steinmuller et al., 1993). There is also more recent research on bacterial endophytes in EP and their ability to enhance the immune system, accounting for the majority of the immunomodulation observed (Pugh et al., 2013; Todd et al., 2015; Haron et al., 2016). Specifically, lipopolysaccharides and Braun-type lipoproteins derived from the bacterial endophytes in EP stimulate the body’s macrophages (Tamta et al., 2008; Pugh et al., 2013), which promotes the production of cytokines (both pro-inflammatory, such as TNF-α and IL-6, and anti-inflammatory, such as IL-10; Burger et al., 1997; Sullivan et al., 2008). The effect of bacterial endophytes is supported by research that demonstrated EP grown in a sterile environment lacked both lipopolysaccharides and macrophage stimulating ability (Todd et al.,
Furthermore, the addition of substances that degrade bacterial lipopolysaccharides and lipoproteins (polymyxin B and lipoprotein lipase) resulted in diminishment of the immunomodulation activity (Tamta et al., 2008). *Echinacea purpurea* is also reported to increase circulating white blood cells (Goel et al., 2005). Therefore, EP can affect the innate immune system (Haron et al., 2016). Considering adaptive immunity, immunoglobulins have also been investigated although there are conflicting results: IgG and IgM levels were reported to increase (Rehman et al., 1999) and decrease (Mishima et al., 2004) in response to Echinacea.

Even though Echinacea has been consumed by humans for medicinal reasons for centuries (Brush et al., 2006), its effects on health and immunity are still not completely understood (Seckin et al., 2018). Furthermore, there is a lack of research on the effects of medicinal plants on young livestock species (Ayrle et al., 2016). The effects of EP supplementation on dairy calves remains under-researched to date and has been largely investigated in combination with other supplements, yielding variable results. Specifically, Ismael et al. (2009) reported that co-administering Echinacea with foot and mouth disease vaccines increased the vaccine’s efficiency in calves. Seckin et al. (2018) reported that supplementation of EP and *Pelargonium sidoides* to calves elicited an immune response, evidenced through increased IgG and γ-interferon levels and upregulation of γ-interferon IL-1-β, IL-2 and TNF-α. Finally, Ayrle et al. (2021) suggested that EP may activate the local enteric immune system, however, they detected mixed results with EP supplementation in calves. Therefore, further investigation with supplementation of EP alone is warranted.
The objective of this study was to determine the effects of EP supplementation on markers of immunity, health, feed intake, and growth of dairy calves. The hypothesis was that EP supplementation, especially to calves during the whole milk-fed period, would reduce inflammation (i.e. lower haptoglobin concentration) and stimulate the immune system (i.e. increased cytokine and white blood cell counts), and consequently, improve the health, intake, and growth of calves compared to calves not receiving EP.

### 2.2 MATERIALS AND METHODS

#### 2.2.1 Animals and Housing

A randomized clinical trial was conducted at a commercial calf rearing facility (Mapleview Agri Ltd., Palmerston, Ontario, Canada), using 3 batches of 80 calves, for a total sample size of 240 male Holstein calves. For each batch, all calves arrived at the facility on 1 d (November 9, 30, and December 21, 2020), sourced from auction barns (n = 103 calves) or directly via 2 drovers (drover 1 = 53 calves, and drover 2 = 84 calves), from approximately 30 local Ontario dairy farms. Each group of 80 calves were housed in structurally identical, but different rooms at the facility. All calves that arrived were enrolled (no calves were excluded at enrollment). All calves had an unknown history, with age estimated to be between 5 to 14 d of life. For each batch, upon arrival, all calves were housed in 1 room (Figure 2.6) that had 4 rows of 20 metal bar-sided stalls (80 stalls total) with a walkway between each row of stalls. Each calf was randomly assigned to a stall (101.60 x 78.74 x 121.92 cm; height x width x length) with
rubber slatted floors and no bedding (as per facility standard) for the milk-fed period (d 0 to 56). Each stall had an opening on the front, which allowed the calves to reach through and consume their milk or solid feed out of a bucket and trough, respectively. The buckets were attached to a metal bar that allowed them to be rotated into position for the calves at milk feeding times, and then rotated beneath the feed trough, out of the way, after milk feeding times. Calves had auditory and visual contact with other calves, however, physical contact was limited due to the metal bar stall sides. For the post-weaning period (d 57 to 77), the gates separating each set of 5 consecutive stalls were removed, which consequently incorporated the previous walkway into the housing area to allow calves in those stalls to co-mingle and have physical contact with each other in a larger space; each group of 5 calves was referred to as a ‘pod’ (101.60 x 198.12 x 396.24 cm; height x width x length). The ventilation system in the rooms consisted of an air inlet on one side and 5 chimney fans to exhaust the air. All study procedures were reviewed and approved by the University of Guelph Animal Care Committee (AUP#4134).

2.2.2 Feeding and Health Management

All calves were offered a skim-milk based milk replacer (MR; 26% crude protein (CP), 17% fat; Mapleview Agri Ltd., Palmerston, Ontario, Canada; Table 2.1). The fat sources in the MR were lard, palm, and coconut. The MR contained no feed additives, including no antimicrobials. Barn staff individually fed the calves MR according to the feeding schedule outlined in Table 2.2, twice a day (at 0600 and 1630 h) by bucket with a floating nipple to drink from. During the first milk feeding, barn staff trained the calves to consume milk out of a bucket
via the floating nipple by allowing the calves to suckle on their fingers and guiding their mouth down onto the nipple. Staff supervised the calves for the first 14 d to try to get as many calves as possible to use the nipple, although some calves preferred to drink straight from the bucket resulting in the nipples being used approximately 90% of the time. All milk rejections were recorded. All calves were completely weaned and received no milk on d 57. For the milk-fed period, calves had ad libitum access to water provided through a nipple water dispenser in each pen, while for the post-weaning period, each pod had ad libitum access to water provided through a water bowl. Ad libitum solid feed was offered from d 1 in a trough, fed per pod, topped up as needed. Intakes were recorded by staff at the end of each wk at 0900 h by doing a weigh back of the feed offered that wk and leftover in the trough. At this time, the trough would be cleaned, orts discarded, and entirely fresh feed offered unless the feed leftover was fresh from the evening before or that day, in which case they would add it back. As outlined in Table 2.2, from wk 1 to 8, calves were offered a calf starter pellet (Mapleview HE Calf Starter; 20% CP; Wallenstein Feed and Supply Ltd., Wallenstein, Ontario, Canada; Table 2.1), and during wk 9, calves were offered a diet that consisted of half calf starter pellet and half calf grower ration (Mapleview Veal Starter 2.5:1; 18% CP; Wallenstein Feed and Supply Ltd., Wallenstein, Ontario, Canada) and 4% chopped straw (The Straw Boss Inc., Mount Elgin, Ontario, Canada), referred to as combo feed (Table 2.1) to transition the calves onto the calf grower feed. In wk 10 and 11, calves were offered the calf grower ration with 4% chopped straw (Table 2.1). Both the calf starter and calf grower feeds contained monensin at 52 mg/kg and 22.3 mg/kg, respectively.
Barn staff performed health assessments daily by walking through the barn and individually assessing each calf. Staff assigned fecal scores to all calves twice a day (0600 and 1600 h) from d 1 to 28. The scoring system was modified from McGuirk (2008): normal consistency feces received a score of 1, semi-formed or pasty feces received a score of 2, runny feces that spread easily received a score of 3, and liquid feces devoid of solid material received a score of 4. For treatment decision purposes (as outlined in Table 2.5) scores 3 and 4 were considered abnormal (neonatal calf diarrhea; NCD), while a score of 4 was considered severe diarrhea (Schinwald et al., 2022). The fecal data were summarized into those categories for analyses. Specifically, the proportion of abnormal fecal scores was calculated for each calf by summing the number of fecal scores that were 3 or 4, dividing by the total number of scores (56), and multiplying by 100 to get a percent. The proportion of severe fecal scores (score or 4) was calculated for each calf using the same method except only summing the number of fecal scores that were 4. Staff assigned respiratory scores to all calves twice a day (also at 0600 and 1600 h) from d 1 to 77 using a scoring system modified from the UC Davis respiratory scoring system (Aly et al., 2020). Calves were assigned points per symptom of Bovine Respiratory Disease (BRD) as follows: 2 points for each eye discharge, cough, rapid/difficult breathing, and rectal temperature > 39.5°C, 4 points for nasal discharge, and 5 points for droopy ears. Rectal temperatures were only taken when the calf had a score of 4 based on the visible symptoms to determine if treatment was needed. All scores were recorded directly into a Microsoft Excel (Microsoft Corp., Redmond, WA, USA) spreadsheet. The respiratory data were summarized into 3 categories: the proportion of respiratory scores with no symptoms of BRD, the proportion of
respiratory scores \( \geq 4 \), and the proportion of respiratory scores \( \geq 5 \). Calves with a score \( \geq 5 \) were diagnosed with BRD (McGuirk and Peek, 2014). Given the low proportion of respiratory scores \( \geq 5 \), the proportion of respiratory scores \( \geq 4 \) was also considered, as this grading has also been used to assess BRD in calves (Love et al., 2014). Treatment of disease was determined based on the health scores and followed a treatment protocol, as outlined in Table 2.5. Following the third treatment for diarrhea and fourth respiratory treatment, no further antimicrobial treatments were administered for diarrhea and BRD, respectively. All medications administered were recorded by barn staff. For each calf mortality, barn staff recorded the suspected cause of death.

Upon arrival to the facility, calves received an intranasal viral vaccine (Inforce-3; Zoetis Canada Inc., Kirkland, Quebec, Canada) and an antimicrobial medication (Draxxin; Zoetis Canada Inc., Kirkland, Quebec, Canada). At 7 d following arrival, calves received an intranasal bacterin (Once-PMH; Merck Animal Health, Kirkland, Quebec, Canada), and at 14 and 28 d following arrival they received an injectable modified-live vaccine (Vista Once; Merck Animal Health, Kirkland, Quebec, Canada).

2.2.3 Treatments Allocation

Calves were randomly allocated to 1 of 3 treatments, which included: 1) receiving 3 g/d of powdered EP extract split over 2 milk feedings from d 14 to 28 (E14; \( n = 80 \) calves), 2) receiving 3 g/d of EP split over 2 milk feedings from d 1 to 56 (E56; \( n = 80 \) calves), and 3) receiving no EP (control group; CON; \( n = 80 \) calves). For the E14 treatment, we were interested
to see if a targeted supplementation of EP from d 14 to 28 following arrival would be as beneficial as supplementing it across the whole milk-fed period. Day 14 to 28 was chosen based on the estimated age of the calves at arrival to the facility (5-14 d of age) and the literature-based time when respiratory illness is first detected in pre-weaned dairy calves (Windeyer et al., 2014; Urie et al., 2018), thus to provide calves with EP while most susceptible to illness. For each room, prior to calf arrival, treatments were randomly allocated to pods using a random number generator in Microsoft Excel (Microsoft Corp., Redmond, WA, USA). This meant there was 25 or 30 calves/treatment in each room, and a total of 80 calves/treatment. The facility manager assigned the treatments to the pods to allow the primary author and fellow researchers to be blinded to the treatments.

At enrollment, following arrival to the facility, calves were weighed and randomly assigned to a stall number between 1 and 80, while ensuring the 3 treatment groups were kept within 0.45 kg of the average arrival BW. The treatments were identified by barn staff using 3 different colored ear tags (white = CON, red = E14 and yellow = E56) to inform staff which treatment to feed each calf. The EP treatments were fed to the calves by the barn staff during milk feedings; the treatments were mixed with 100 g of MR and added directly to the tank mix of milk. The staff did not know what the supplement was, nor the anticipated outcome, and had a busy schedule with multiple trials running, and therefore bias was not believed to be an issue for any of the data collected. The same number generator in Microsoft Excel (Microsoft Corp., Redmond, WA, USA) was used to select a subset of calves (13 calves/treatment in each room, 39
calves/treatment, total of 117 calves) for collection of additional measures including rectal temperature, blood haptoglobin, cytokine (IL-10, IL-6 and TNF-α) concentrations, and white blood cell (WBC) count and differentiation. Two to 3 calves were randomly selected from each pod to have them as equally distributed among the pods as possible.

2.2.4 Sample Size Calculation

Sample size calculations were conducted through a power analysis (Morris, 1999; Hintze, 2008) to ensure an adequate number of experimental units (calves) per treatment. It was estimated that a calf in the control group would have a pre-weaning average daily gain (ADG) of 0.60 kg/day (SD = 0.2 kg/day; CV=33%). With a 10% predicted difference, calves on the EP treatments would have an ADG of 0.66 kg/day (SD = 0.2 kg/day). Using 95% confidence interval and 80% power, a minimum sample size of 80 calves per treatment group or 240 calves total was determined to be required. It was estimated that the incidence of calf diarrhea and respiratory disease in the control group would be 20% and 30% (SD = 10%), based on historical facility data. We estimated that 80 calves per treatment group (n = 240 total), at 95% confidence interval and 80% power, would allow us to detect a 15 percentage point difference in incidence of these health conditions.

2.2.5 Measurements and Sample Collection

Barn staff (a total of 6 individuals) collected all measurements, except blood samples, rectal temperatures, and hip heights, which were collected by the primary author with the
assistance of fellow researchers (a total of 4 individuals). Of the 6 barn staff, only 2 did health scoring.

Blood sampling was completed on d 1, 14, 28, and 57 in the morning. On d 1, blood was collected from all calves into a 10 mL vacutainer red top glass blood collection tube (BD, Franklin Lakes, New Jersey, USA), while an additional sample was collected from the subset calves into a 10 mL vacutainer lavender top plastic blood collection tube with K2 EDTA (BD, Franklin Lakes, New Jersey, USA). On d 14, 28 and 57, the same 2 samples were collected from subset calves only. All blood samples were taken from the jugular vein using a 20 gauge x 2.54 cm vacuette multiple use drawing needle (greiner BIO-ONE, Kremsmünster, Austria) and a vacutainer tube holder (BD, Franklin Lakes, New Jersey, USA). The sampler gently restrained the calf with their body in the calf’s stall for collection. Lavender top tubes were gently inverted approximately 8 times immediately after collection to mix the blood with the K2 EDTA. Blood tubes were placed on ice in an insulated cooler after collection. Upon arrival at the University of Guelph, all lavender top tubes were delivered to the Animal Health Laboratory (AHL; University of Guelph, Guelph, Ontario, Canada) where they were prepared and analysed to determine the total WBC count and differentiation (segmented neutrophil, band neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts). All red top tubes, having had sufficient clotting time during the travel back to the University of Guelph, were placed in a centrifuge (Sorvall Legend RT, Germany) upon arrival and centrifuge cooling was completed. The samples were then centrifuged at 1000 x g for 10 minutes at 4°C to obtain serum. Using disposable
plastic pipets (Fisher Scientific, Mississauga, Ontario, Canada), serum was aliquoted as follows: on d 1, a few drops of serum were placed on a handheld digital refractometer (MISCO, Ohio, USA) to determine serum total protein (STP) and confirm passive immunity status, with failed transfer of passive immunity (FTPI) identified for STP values < 5.2 g/dL (Renaud et al., 2020). The rest of the serum for the non-subset calves was discarded, while for all 4 blood sampling days, the remaining serum for the subset calves was divided in triplicate (2 for analyses, 1 spare) into 0.5 mL snap cap tubes (Fisher Scientific, Mississauga, Ontario, Canada) and frozen at -20°C. Later, serum samples from each subset calf were delivered to the AHL (in an insulated container with freezer packs) where they were analysed for haptoglobin content (g/L), using an in-house assay based on the work of Makimura and Suzuki (1980) and Skinner et al. (1991). At the end of the trial, serum samples were also sent to Eve Technologies Corp. (Calgary, Alberta, Canada; in an insulated container with dry ice), where they were analyzed for cytokines. A multiplexing analysis was performed using the Luminex 200 system (Luminex, Austin, Texas, USA). Three markers were simultaneously measured in the samples using a Custom Bovine Cytokine 3-Plex Magnetic Bead Assay (MilliporeSigma, Burlington, Massachusetts, USA) according to the manufacturer's protocol. The 3-plex consisted of IL-6, IL-10, and TNF-α. Assay sensitivities of these markers range from 0.57 to 10.88 pg/mL for the 3-plex. The intra-assay %CV for all 3 cytokines was < 10%, while the inter-assay %CV was < 15% for IL-6 and TNF-α, and < 10% for IL-10. Assessed blood parameters were chosen based on known EP impacts reported in the literature to date (described above), as well as budget and laboratory constraints.
No studies were identified that assessed acute phase proteins such as haptoglobin; we chose to assess haptoglobin in this study since EP has been reported to affect inflammation.

On d 1, 14, 28, and 57, during blood sampling, a rectal temperature (Accuflex 10 Flexible Digital Thermometer, Montreal, Quebec, Canada) was also taken and recorded on subset calves.

The MR refusals were determined by measuring any refused MR at each feeding and recorded for the purposes of monitoring health and calculating the total MR intake/calf at the end of the trial (by subtracting it from the total kg of MR offered). The solid feed refusals were determined at the end of each wk by weighing the orts and recorded to later calculate weekly solid feed intakes/pod (by subtracting it from the total kg of feed offered that wk). Samples of the MR and solid feed rations were taken biweekly. These samples were frozen at -20°C for later analysis. Samples were later thawed and individually placed in a drying oven at 60°C for 48 h to determine the dry matter (DM) content. Dry matter intake (DMI) was calculated by multiplying the kg of solid feed consumed by the DM of the corresponding MR and/or solid feed ration sample for that wk of data. Samples of solid feed were ground through a 1-mm sieve (Model 4 Wiley Laboratory Mill, Thomas Scientific, Swedsboro, New Jersey, USA) and were sent to A & L Canada Laboratories Inc. (London, Ontario, Canada) for chemical composition analyses. Calf starter, combo and finisher feed samples were analyzed for ash (550°C; AOAC International, 2000: method 942.05), CP (N x 6.25; AOAC International, 2000: method 990.03; Leco FP-628 Nitrogen Analyzer, Leco, St. Joseph, MI), Acid Detergent Fiber (ADF; AOAC International, 2000: method 973.18), Neutral Detergent Fiber (NDF) with heat-stable α-amylase and sodium
sulfite (AOAC International, 2000: method 2002.04), starch (heat-stable amylase and amylglucosidase; AOAC International, 2000: method 996.11), and crude fat (AOAC International, 2000: method 920.39; Ankom XT15). Milk replacer samples were also analyzed at A & L Canada Laboratories Inc. for CP (N x 6.25; AOAC International, 2000: method 990.03; Leco FP-628 Nitrogen Analyzer, Leco, St. Joseph, MI), crude fibre (AOAC International, 2000: method Ba 6a-05; Ankom Bag Technology), and fat (total fat by acid hydrolysis using the ANKOM HCl Hydrolysis System; AOAC International, 1995: method 954.02 Section 4.5.02). Metabolizable energy was calculated for all samples using National Research Council (NRC, 2001) equations, and then the average ME was calculated for the MR and each of the solid feed rations. The weekly DMI values were then multiplied by the average ME of the corresponding MR and/or solid feed ration sample for that wk of data to determine ME intake. For the milk-fed weeks, the ME intake from the MR and solid feed were summed to get a total. The total weekly ME intakes were then divided by pod gain to determine pod feed conversion rate (FCR) on a ME basis.

Samples of the EP were taken at the beginning of each month of the trial and frozen at -20°C until the end of the trial. These samples were shipped to Eurofins Food Integrity and Innovation lab (Madison, Wisconsin, USA), where they were analysed for % moisture (United States Pharmacopeia, 43rd Revision - National Formulary 38th Edition. USP Convention. Rockville, MD, 2019, Modified, Madison, Wisconsin, USA) to determine DM content, aerobic plate count (standard plate count; CFU/g; FDA BAM Ch.3, AOAC 966.23, CMMEF Ch. 8, EML
New Berlin, USA), and phenolic content including chlorogenic acid, cichoric acid, echinacoside, caftaric acid, and total phenolics (ppm; Sakakibara et al., 2003, Bauer and Wagner, 1991, Madison, Wisconsin, USA). Averages were calculated and reported in Table 2.3.

Calf BW was measured at 0900 h on arrival (d 0), and on d 7, 14, 21, 28, 35, 42, 49 56, 63, 70 and 77 using a calibrated platform scale (Tru-Test weigh platform; Tru-Test DATAMARS, Mineral Wells, Texas, USA). Hip height was measured in the morning on d 1, 14, 28, and 57 of the trial.

2.2.6 Statistical Analyses

All statistical analyses were conducted using SAS 9.4 software (SAS Institute Inc., 2013), except diarrhea and BRD diagnoses and corresponding medication administrations, which were analyzed using Stata 16 (StataCorp LP, College Station, Texas). All data were imported into the statistical software programs from Microsoft Excel (Microsoft Corp., Redmond, WA, USA), where it was checked for completeness. In SAS, data were assessed for normality using the UNIVARIATE procedure. When parameters did not meet the assumptions of normality, a log10 transformation was used and successfully transformed the data to a normal distribution for the analysis. Transformed variables included: monocytes, IL-6, IL-10, and TNF-α, abnormal and severe fecal scores, rectal temperature, electrolyte doses, and the proportion of respiratory scores ≥ 4 (milk-fed and post-wean periods). The proportion of respiratory scores ≥ 5 was not able to be
normalized, and thus was categorized and presented as the risk of having BRD (1 = the calf had at least 1 respiratory score \( \geq 5 \), 0 = the calf had no respiratory scores \( \geq 5 \)).

Multivariable regression analyses were conducted for all parameters except medication administrations. The GLIMMIX procedure was used for all linear regression repeated measures analyses and logistic regression models, while the MIXED procedure was used for all linear models not containing repeated measures. For all repeated measures analyses with unequal time spacing, the covariance structure cs was used. For the repeated measures analyses with equal time spacing, the covariance strictures cs, csh, arh(1), ar(1), and un were tested and the one with the lowest BIC value was used for the analysis. The variables analyzed with logistic regression were the risk of BRD and mortality for both the milk-fed and post-wean periods. For each model, room was a block, kept constant as a fixed effect in each model to account for potential differences between the rooms at the facility. The covariates tested in each model were the categorical variables of FTPI (yes or no; 31.7 % of calves had FTPI) and source (auction, drover 1, or drover 2), and the continuous variable arrival BW (average = 47.6 kg, minimum = 42.2 kg, maximum = 53.1 kg). The d 1 rectal temperatures were removed from the data and used as an additional covariate, kept constant as a fixed effect, for the rectal temperature analysis. Failed transfer of passive immunity and arrival BW were correlated, so if both were tendencies or significant for a particular parameter, the more biologically related covariate was chosen to remain in the model. The baseline parameters were compared between treatments to ensure they did not vary. This was done using linear (arrival BW and STP) and logistic (FTPI) regression
models and a chi square test (source). For any variables that were tendencies or significant, the interaction of it with treatment was assessed. For the repeated measures analyses, the interaction of treatment and day was included. A manual backward stepwise process was used to eliminate variables that were not tendencies or significant in the models, although treatment, day/wk (for the repeated measures analyses), and room were kept in the models regardless of their outcome. The calf was the experimental unit in all analyses, except grain consumption and FCR, since grain consumption was measured at the pod level. Significance was declared at $P \leq 0.05$, and tendencies at $0.05 < P \leq 0.10$.

In Stata 16 (StataCorp LP, College Station, Texas), Cox proportional hazard models were created to access the effect of treatment on medication administration for diarrhea and BRD. A logistic regression model was used to determine the effect of treatment on recurrent medication administrations. Note that data from both the milk-fed and post-wean periods were included in determining the risk of receiving the respiratory treatments. Similar model building strategies were used, as outlined above, and model fit was determined by evaluating the assumption of proportional hazards in the hazard models.

2.3 RESULTS

The baseline parameters did not differ between treatment groups, including arrival BW ($P = 0.76$), STP ($P = 0.34$), FTPI ($P = 0.91$), and source ($P = 0.94$).
2.3.1 Blood

Calves in the E14 and E56 treatments tended to have lower levels of haptoglobin than CON calves (Table 2.4). Calves in the E56 treatment tended to have less segmented neutrophils than CON calves (Table 2.4). Calves in the E14 treatment tended to have more lymphocytes, while E56 calves had more lymphocytes than CON calves (Table 2.4). Calves in the E14 treatment tended to have a lower segmented neutrophil/lymphocyte ratio (N:L) ratio, while E56 calves had a lower N:L ratio than CON calves (Table 2.4). There was a treatment × arrival BW interaction for eosinophils (P = 0.005; Figure 2.1). Calves in the CON treatment had increased eosinophil counts with increased arrival BW, whereas calves in the E14 and E56 treatments had consistent eosinophil counts regardless of arrival BW.

There was no detected effect of the EP treatments on total WBC, band neutrophil, monocyte, and basophil (Table 2.6) counts, nor IL-10, IL-6, and TNF-α levels (Table 2.7).

2.3.2 Health

There tended to be a treatment × FTPI × d interaction (P = 0.09) for rectal temperature; on d 14, of the calves with FTPI, E14 calves tended to have a lower temperature than CON calves (P = 0.10; Figure 2.2). Additionally, on d 28, of calves without FTPI, E14 calves had a higher temperature than CON calves (P = 0.02), and E56 calves tended to have a higher temperature than CON calves (P = 0.08; Figure 2.2).
In the milk-fed period, there was a treatment × source interaction (P = 0.009) for the proportion of respiratory scores with no symptoms of bovine respiratory disease; of calves sourced from auction, E14 calves tended to have a higher proportion of scores with no symptoms of BRD than CON calves (P = 0.08) and E56 calves had a higher proportion of scores with no symptoms of BRD than CON calves (P = 0.03; Figure 2.3). Conversely, of calves sourced from drover 2, E14 calves tended to have a lower proportion of scores with no symptoms of BRD than CON calves (P = 0.08) and E56 calves had a lower proportion of scores with no symptoms of BRD than CON calves (P = 0.01; Figure 2.3). In the milk-fed period, there was no detected effect of the EP treatments on the proportion of abnormal nor severe fecal scores, electrolyte doses (Table 2.8), the risk of receiving diarrhea treatment (Table 2.9), the proportion of respiratory scores ≥ 4 (Table 2.8), the risk of BRD, and the risk of mortality (Table 2.10).

In the post-wean period there was a treatment × source interaction (P = 0.01) for the proportion of respiratory scores ≥ 4; of calves sourced from auction, E14 calves tended to have a lower proportion of respiratory scores ≥ 4 than CON calves (P = 0.06; Figure 2.4). Of calves sourced from drover 1, E14 calves had a higher proportion of respiratory scores ≥ 4 than CON calves (P = 0.02) and tended to have a higher proportion of respiratory scores ≥ 4 than E56 calves (P = 0.10; Figure 2.4). Of calves sourced from drover 2, E14 calves had a higher proportion of respiratory scores ≥ 4 than CON calves (P = 0.05; Figure 2.4). In the post-wean period, there was no effect of the EP treatments detected on the proportion of scores with no symptoms of BRD (Table 2.13), the risk of BRD, the risk of mortality (Table 2.14), and the risk
of receiving respiratory treatment (Table 2.15). Throughout the whole trial, a total of 22 calves (9.17%) died.

2.3.3 Intake and Growth

In the milk-fed period, there was no effect of the EP treatments detected on any of the intake nor growth parameters, including MR intake, grain intake, FCR (Table 2.11), hip height, weekly BW, and ADG (Table 2.12). In the post-wean period, there was a treatment \( \times \) arrival BW interaction \((P = 0.004)\) for post-wean period weekly BW; of calves with heavier arrival BW, E56 calves had higher average post-wean weekly BW than CON calves \((P = 0.006;\) Figure 2.5). There was no detected effect of the EP treatments on grain intake, ADG, and FCR (Table 2.13).

2.4 DISCUSSION

This study is a thorough investigation of the effects of supplementing EP to dairy calves. *Echinacea purpurea* supplementation was associated with reduced haptoglobin levels, reduced segmented neutrophil counts and a lower N:L ratio, as well as increased lymphocyte counts. Additionally, EP supplementation was associated with a higher average post-wean body weight among calves with heavier arrival BW. Effects were detected to a greater extent in E56 rather than E14 calves. Supplements are a growing area of research, and rightfully so, as they have the potential to support dairy calves in various ways, including immunity, health, growth and welfare, and can be easily implementable on farm. Calf-hood success is vital to the future productivity and sustainability of the dairy industry.
It was hypothesized that EP supplementation, especially for the whole milk-fed period (E56), would be associated with reduced inflammation (reduced haptoglobin) and stimulated immunity (increased cytokines and white blood cell counts) due to EP’s active components, which would in turn minimize illness. In support of this, haptoglobin tended to be lower in the E14 and E56 calves, indicative of lower inflammation (Murray et al., 2014). To our knowledge, no studies have assessed haptoglobin in response to only EP supplementation. In support of our finding, Fararh et al. (2017) reported that diseased calves supplemented with Tulathromycin and an essential oil mixture, which included EP, had lower haptoglobin levels than diseased calves treated with just Tulathromycin alone. Given the combination of supplements provided in that study, one cannot conclude their results are related solely to the EP. For this reason, in the following discussion of results from other EP supplementation studies, only those results from supplementation of EP alone are presented, unless otherwise stated (i.e. EP was supplemented in combination with other products).

A tendency for fewer segmented neutrophils in the E56 calves would be indicative of lesser infection and/or inflammation (Doherty et al., 2007; Cuevas-Gomez et al., 2020). This is further evidence (in addition to reduced haptoglobin) of reduced inflammation in E56 calves. Although there are conflicting results in the literature regarding the effect of EP supplementation on neutrophil counts, including decreased levels in rabbits (Ahmed et al., 2008) and chickens (Nosrati et al., 2017), increased levels in chickens (Dehkordi et al., 2011; Enany et al., 2017), and no detected effect in pigs (Maass et al., 2005), dogs (Torkan et al., 2015), and calves (Ayrle
et al., 2021). Greater lymphocytes were detected in calves supplemented with EP, especially those receiving it throughout the whole milk-fed period, indicative of an immune system more prepared to defend the body against pathogens (Orakpoghenor et al., 2019), supporting our hypothesis of stimulated immunity. Similarly, EP supplementation for a longer duration, compared to a shorter duration and control, was associated with higher lymphocyte count in chickens (Dehkordi et al., 2011). Likewise, supplementation of EP has been associated with increased lymphocyte counts in dogs (Torkan et al., 2015) and chickens (Enany et al., 2017; Nosrati et al., 2017). Conversely, some researchers did not detect any effect of EP supplementation on lymphocyte counts in pigs (Maass et al., 2005), chickens (Gharieb and Youssef, 2014), and calves (Ayrle et al., 2021). The N:L ratio has been used as a measure of illness and stress in humans and animals. In fact, it was reported to be higher in humans with advanced or aggressive cancer (Guthrie et al., 2013) and severe cases of Covid-19 (Zheng et al., 2020), and in sick and/or presumably stressed calves (Doherty et al., 2007; Cuevas-Gomez et al., 2020). A lower N:L ratio was detected in calves supplemented with EP, especially those receiving it throughout the whole milk-fed period, indicative of less illness and/or stress. Similarly, Nosrati et al. (2017) detected a lower N:L ratio in chickens supplemented with EP. Although, other researchers detected no effect of EP supplementation on the N:L ratio including in chickens (Gharieb and Youssef, 2014; Jahanian et al., 2017) and calves (Ayrle et al., 2021). Overall, the lower haptoglobin, segmented neutrophils, and N:L ratio, as well as higher lymphocytes associated with the treatments receiving EP supplementation in the present study are likely due to the active components in the EP, namely the phenolics, alkamides, and
polysaccharides, causing antioxidant, anti-inflammatory, and immune stimulatory effects, respectively. Comparing the results of this study to Ayrle et al. (2021), the EP supplemented in our study had higher concentrations of phenolics. For example, considering cichoric acid – the primary phenolic in EP (Dalby-Brown et al., 2005) - the EP in our study contained 2332.5 + 59.10 ppm, while Ayrle et al. (2021) reported the EP they supplemented only contained 313.8 ppm. Cichoric acid in the diet produces antioxidant activity, which supports health and a properly functioning immune system (Puertollano et al., 2011). Therefore the phenolic content difference is likely a major reason behind the differences detected. We were unable to locate the resources to determine the alkamide and polysaccharide content in the EP supplemented, which was also not reported by Ayrle et al. (2021) for their EP, although those components were likely to vary as well, which would further impact the differences in results.

Various blood parameters did not, however, align with our hypothesis. Of calves with heavier arrival BW, E14 and E56 calves’ eosinophil counts remained relatively consistent while CON calves’ eosinophil counts were elevated. It is possible that a heavier arrival BW in addition to EP supplementation was favorable for health, preventing an increase in eosinophils. However, Ayrle et al. (2021) detected lower eosinophil counts in calves receiving a low EP dose compared to control, but no difference for calves receiving a high dose. Conversely, Ahmed et al. (2008) reported that rabbits supplemented with a high dose of EP had increased eosinophils compared to lower doses and their control. In general, most researchers have reported no effects
of EP supplementation on eosinophil count, including in pigs (Maass et al., 2005) and chickens (Dehkordi et al., 2011; Gharieb and Youssef, 2014).

Despite the expectation that WBC, band neutrophil, basophil, and monocyte counts would all be lower in E14 and E56 calves, no differences were detected. The increase in lymphocytes and decrease in neutrophils associated with EP supplementation in the present study may have eliminated a change in WBC count. Similarly, other researchers did not detect an effect of EP supplementation on WBC count in pigs (Maass et al., 2005), chickens (Gharieb and Youssef, 2014), or calves (Ayrle et al., 2021). However supplementation of EP has been associated with an increased WBC count in various studies, including in chickens (Dehkordi et al., 2011; Enany et al., 2017) and dogs (Torkan et al., 2015). In those studies, such an increase may be explained by the EP activating the immune system (as illness would), causing an increase in WBC count to prepare the body to fight infection. To our knowledge there is a lack of data available in the literature on the effect of EP supplementation on band neutrophils and basophil counts. Like our results, Torkan et al. (2015) also did not detect any effect of EP supplementation on band neutrophil counts in dogs, and Maass et al. (2005) and Torkan et al. (2015) also did not detect any effect of EP supplementation on basophil counts in pigs and dogs, respectively. Alternatively, Ahmed et al. (2008) reported decreased basophil counts in rabbits. Similar to our results, other researchers did not identify any effects of EP supplementation on monocyte count in chickens (Gharieb and Youssef, 2014; Enany et al., 2017) and calves (Ayrle et al., 2021). Greater and decreased monocyte counts, however, were reported in chickens
(Jahanian et al., 2017) and rabbits (Ahmed et al., 2008), respectively. Eosinophils, band neutrophils, and basophils are all found in low quantities in calves (Knowles et al., 2000), which may explain the variable and/or lack of detected effects on these measures.

Contrary to our hypothesis, especially given the noted effects on haptoglobin and the N:L ratio, IL-10, IL-6, and TNF-α levels, which are known to regulate inflammation, did not vary with EP treatment. A likely explanation is that the EP supplemented had a low aerobic plate count (<10.0 + 0.00 CFU/g), as it is the bacterial endophytes that are reported in the literature to have the greatest effect on cytokines, as noted above. In general, IL-10 levels have not been well investigated in calves, while IL-6 and TNF-α have been more thoroughly investigated in calves, with higher levels associated with illness in several studies, including pneumonia (Akgul et al., 2019) and neonatal calf diarrhea (Beheshtipour and Raeeszadeh, 2020). Considering EP supplementation studies, cytokine results are variable. Schwarz et al. (2002) and Ayrle et al. (2021) did not detect any effect of EP supplementation on TNF-α levels in humans and TNF-α mRNA in calves, respectively. However a low dose of EP supplementation was associated with lower IL-6 levels and a high dose of EP supplementation was associated with lower TNF-α levels in rodents (Yu et al., 2013). Conversely, in vitro human macrophages cultured in EP produced higher levels of IL-10, IL-6, and TNF-α than human macrophages cultured in a control medium (Burger et al., 1997). Likewise, Seckin et al. (2018) detected greater upregulation of TNF-α gene expression in calves receiving EP and Pelargonium sidoides. Due to the high variability in results, more research is needed to elucidate the impact of EP supplementation on
cytokine levels. It is noteworthy that the majority of studies assessing the impact of EP supplementation were focused on blood markers and performance (intake and growth) measures but did not report on animal health outcomes.

Some, although not all, results aligned with the hypothesis that EP supplementation would improve calf health. Of those calves with FTPI, E14 calves tended to have a lower temperature on d 14 than CON calves. The calves received the EP at 0600 h, while the temperatures were taken throughout the morning depending on the calves’ location in the room. It is possible that the introduction of EP resulted in a lower rectal temperature in these immune compromised calves due to its anti-inflammatory properties. On d 28, of calves without FTPI, EP supplemented calves had a higher rectal temperature, especially those receiving it for just 2 wk. This was unexpected, because a high rectal temperature is a well understood symptom of illness (Hart, 1988), and therefore was expected to be lower in calves receiving EP, especially as the trial proceeded to give the EP time to take effect. It is possible the EP can cause a higher temperature by stimulating the immune system. Ayrle et al. (2021) reported that EP resulted in a higher rectal temperature in calves on low and high EP supplementation treatments compared to control, although on different days throughout the trial. Evidently more investigation is needed.

Contrary to our expectation, EP supplementation did not result in any improvement in fecal scores or reduced diarrhea treatment. Ayrle et al. (2021) reported a low dose of EP supplementation reduced the number of days with diarrhea in calves by 44%, although a high
dose had no effect. The effects of EP on digestive health are lacking in the literature and require more investigation.

In the milk-fed period, of calves sourced from auction, EP supplementation was associated with a higher proportion of scores with no symptoms of BRD, especially in calves receiving it throughout the whole milk-fed period. The blood parameters indicative of improved immunity associated with the E14 and E56 treatments likely contributed to this result. It could be assumed that calves sourced from auction incurred more stress than those sourced from the drovers, and research underlines that stress can negatively affect the immune system (Hulbert and Moisa, 2016). Perhaps the effects of EP on health can be more evident in immune compromised and/or stressed animals. Surprisingly, in the milk-fed period, of calves sourced from drover 2, EP supplementation was associated with a lower proportion of scores with no symptoms of BRD, especially in calves receiving it throughout the whole milk-fed period. This was an unexpected finding, as the opposite result was reported in auction calves (as discussed above), yet no differences in blood markers, health, intake, or growth parameters were detected between calves sourced from drover 2 and auction. Despite pre-weaning differences, in the post-wean period, there was no effect of the EP treatments detected on the proportion of scores with no symptoms of BRD.

While no differences were detected in the milk-fed period, in the post-wean period, of calves sourced from auction, E14 calves tended to have better respiratory health (i.e. a lower proportion of respiratory scores ≥ 4) than CON calves, while, of calves sourced from drover 2,
E14 calves had worse respiratory health than CON calves. Again, since there were no differences detected in blood markers, health, intake, or growth parameters between calves sourced from drover 2 and auction, it is unknown why the opposite result occurred in auction versus drover 2 calves. Of calves sourced from drover 1, E14 calves had the worst respiratory health. Again, worse respiratory health in E14 calves compared to CON calves was not expected. Despite this, a higher proportion of respiratory scores ≥ 4 in calves sourced from drover 1 is not surprising - for as compared to auction calves, drover 1 calves had more diarrhea, a lower lymphocyte count, higher IL-6 levels, less MR intake, and a 4 × greater risk of mortality. Similarly, Ayrle et al. (2021) reported a low dose of EP supplementation to calves resulted in an increased number of days with respiratory disease during a follow-up period, although a high dose did not vary from control. Possibly, a shorter duration and/or lesser amount of EP supplementation can still stimulate the immune system in seemingly beneficial ways, as shown by some of the blood results in the present study, although is not enough to combat illness. Furthermore, such EP supplementation combined with immune system activation from disease may overload the calf’s immune system (Aucoin et al., 2020), negatively impacting their ability to fight disease.

Echinacea supplementation had no detected effect in either the milk-fed or post-wean periods on the risk of BRD or risk of receiving respiratory treatment. This does not align with the blood parameters indicative of improved immunity, although is not surprising due to the conflicting respiratory score results. The minimal respiratory health benefits detected were unexpected because there has been a lot of research focused on EP as a supplement to support
respiratory health. Research in humans is largely focused on the use of EP as a treatment rather than a preventative supplement. Taylor et al. (2003) reported treatment with EP did not improve symptoms of upper respiratory tract infections in children, while Weber et al. (2005) reported EP may reduce the risk of subsequent upper respiratory tract infections in children. Likewise, Aucoin et al. (2020) reported it may improve symptoms of acute respiratory infections and the common cold, especially when taken at the first sign of infection. Despite inconclusive results, there is a large consensus that EP consumption may have respiratory benefits; in fact, the Covid-19 pandemic brought even more attention to this herb through investigations on its efficacy in the treatment of Covid-19 infections (Aucoin et al., 2020). Additionally, a review by Ayrle et al. (2016) concluded that EP was 1 of 3 medicinal plants (out of 30 evaluated) with the most promise for the prevention and/or treatment of respiratory diseases in calves and piglets. Overall, the effects of EP supplementation on respiratory health, especially in calves, is lacking in the literature and requires more investigation, ideally including lung ultrasound to truly identify BRD status.

Contrary to expectation, EP supplementation had no detected effect on the risk of mortality in either the milk-fed or post-wean periods. To our knowledge, there is no other work on EP supplementation on mortality rates in calves for comparison. That said, Enany et al. (2017) reported that during an induced E.coli infection in chickens, EP reduced morbidity and mortality rates, and Ahmed et al. (2008) reported lower mortality in rabbits supplemented with EP.
It was hypothesized that EP supplementation, through improving immunity and health, would allow more energy to be devoted to growth and, therefore, result in improved performance parameters. Only one such effect was detected: of calves with heavier arrival BW, those receiving EP throughout the whole milk-fed period had a higher post-wean weekly BW. It is sensible that calves with higher arrival BW would be predisposed to having larger BW long term. Additionally, E56 calves had the lowest neutrophil count and N:L ratio and greatest lymphocyte count supporting inflammation reduction, immunity stimulation, and possibly improved affective state, all of which would likely support further growth post-weaning.

Intake and growth in animals in response to EP supplementation has been thoroughly investigated in the literature. Like the large majority of our results, in many studies there was no effect of EP supplementation detected on performance parameters, including feed intake in rabbits (Ahmed et al., 2008) and chickens (Gurbuz et al., 2010; Dehkordi et al., 2011), BW gain in pigs (Maass et al., 2005) and chickens (Dehkordi et al., 2011), and FCR in piglets (Maass et al., 2005) and chickens (Jahanian et al., 2017). Although in some studies there were reported improvements in performance parameters associated with EP supplementation, including increased feed intake in \textit{E. coli} challenged chickens (Gharieb and Youssef, 2014), BW gain in chickens (Gharieb and Youssef, 2014; Nosrati et al., 2017; Enany et al., 2017), and lower FCR in pigs (Maass et al., 2005), rabbits (Ahmed et al., 2008) and chickens (Dehkordi et al., 2011). It is presumed that the active components in the EP supported growth, as hypothesized in the present study. While only noted in a few studies, there were worse outcomes in performance parameters
associated with EP supplementation, Gurbuz et al. (2010) reported lower ADG and higher FCR in chickens supplemented with EP. There are many reasons for the variability in results across studies, including 1) the EP product used varied: handling factors such as growing medium, age of plant at harvest, part of plant used (root, aerial, or whole), and method of extraction affect the active component profile of the EP (Dehkordi et al., 2011; Haron et al., 2019); 2) the dose amounts and duration used varied, and 3) housing conditions and animal care varied, which are factors known to impact immunity and health. Additionally, there is minimal calf research on the effects of EP supplementation. Most research was conducted with chickens, which are less ideal comparisons. Of the calf studies available, and in many of the studies with other animal species, EP has been often supplemented in addition to other products; as challenging as it is to demonstrate the activity of a single feed additive, it is even more complex when a combination is used.

This study had a few notable limitations. First, health history and care (birthing management, vaccinations, colostrum and milk/grain feeding, housing, etc.) of the calves prior to arrival to the calf rearing facility for enrollment in the study was unknown and likely variable depending on the farm they came from. Also, the exact age of each calf was unknown; calf immunity and health can vary depending on age, therefore variable ages among calves could affect the results. Additionally, the details listed in the previous paragraph for the EP product used were unknown. We were unable to follow the calves past 77 d to see if there were long term effects. Due to time and resource limitations, we were unable to enroll a larger number of calves.
in order to investigate more treatment regimes (dosage of EP/d and duration of supplementation) in order to identify the most effective. Due to facility design, calves were fed solid feed by pods rather than individually; the latter method would have been superior to be able to report solid feed results (intake and FCR) at the calf level. Finally, EP was only supplemented in the MR during the milk-fed period and was unable to be supplemented at the calf level post-weaning due to group housing, although it would have been interesting to see if longer term supplementation through top dressing it on calf starter post-weaning had any effects.

2.5 CONCLUSION

Supplementation of EP was associated with blood markers indicative of reduced inflammation and a stimulated immune system, particularly when fed throughout the whole milk feeding period, although minimal beneficial effects were detected on health and performance. Importantly, any negative effects identified were covariate dependent and, therefore, require more investigation. More research on EP supplementation to dairy calves is recommended to confirm and support its effects and identify the best EP product, dose and duration of supplementation to maximize its benefits.

2.6 ACKNOWLEDGEMENTS

Thank you to all the staff at Mapleview Agri Ltd. (Palmerston, ON, Canada) who managed the calves holding a vital role in this study. Thank you to Rachel Genore-Roche of ACER Consulting (Guelph, ON, Canada) for helping with blood collection, and to Kaitlyn Dancy, Sarah
Parsons, Catalina Wagemann Fluxa, and Anna Schwanke of the University of Guelph (Guelph, ON, Canada) for their assistance with sample collection and processing. Funding and research support were received through the Ontario Agri-Food Innovation Alliance Research Program of the University of Guelph and the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA; Guelph, ON, Canada), as well as from contributions from Mapleview Agri Ltd. The authors have not stated any conflicts of interest.
Table 2.1. Ingredient and chemical composition (mean ± SD) of the calf starter, combo feed\(^1\), calf grower feed\(^1\) and milk replacer fed to all calves.

<table>
<thead>
<tr>
<th>Ingredient, % in ration</th>
<th>Calf starter feed(^2)</th>
<th>Combo feed</th>
<th>Calf grower feed(^3)</th>
<th>Milk replacer(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf starter pellet(^5)</td>
<td>100</td>
<td>48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calf grower pellet</td>
<td>-</td>
<td>13.7</td>
<td>27.5</td>
<td>-</td>
</tr>
<tr>
<td>Whole corn</td>
<td>-</td>
<td>33.8</td>
<td>67.5</td>
<td>-</td>
</tr>
<tr>
<td>Chopped Straw(^6)</td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Oil</td>
<td>-</td>
<td>0.5</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>DM, % DM</td>
<td>88.9 ± 0.56</td>
<td>89.0</td>
<td>89.8</td>
<td>98.0 ± 0.37</td>
</tr>
<tr>
<td>CP, % DM</td>
<td>20.3 ± 0.32</td>
<td>18.5</td>
<td>18.1</td>
<td>24.6 ± 0.49</td>
</tr>
<tr>
<td>NDF, % DM</td>
<td>22.7 ± 1.68</td>
<td>20.4</td>
<td>15.7</td>
<td>-</td>
</tr>
<tr>
<td>ADF, % DM</td>
<td>14.4 ± 1.10</td>
<td>12.0</td>
<td>6.9</td>
<td>-</td>
</tr>
<tr>
<td>Crude Fibre, % DM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.4 ± 0.33</td>
</tr>
<tr>
<td>Ash, % DM</td>
<td>6.7 ± 0.24</td>
<td>7.5</td>
<td>5.7</td>
<td>7.2 ± 0.15</td>
</tr>
<tr>
<td>NFC, % DM</td>
<td>45.5 ± 1.68</td>
<td>49.6</td>
<td>54.8</td>
<td>-</td>
</tr>
<tr>
<td>Starch, % DM</td>
<td>28.9 ± 0.51</td>
<td>32.6</td>
<td>39.4</td>
<td>-</td>
</tr>
<tr>
<td>Fat, % DM</td>
<td>4.1 ± 0.34</td>
<td>5.5</td>
<td>6.8</td>
<td>16.4 ± 0.70</td>
</tr>
<tr>
<td>Lactose, % DM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>51.9 ± 0.33</td>
</tr>
<tr>
<td>Ca, % DM</td>
<td>0.9 ± 0.10</td>
<td>0.9</td>
<td>1.2</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>P, % DM</td>
<td>0.5 ± 0.02</td>
<td>0.4</td>
<td>0.5</td>
<td>0.8 ± 0.01</td>
</tr>
<tr>
<td>Na, % DM</td>
<td>0.4 ± 0.06</td>
<td>0.4</td>
<td>0.4</td>
<td>0.5 ± 0.04</td>
</tr>
<tr>
<td>ME(^{10}), Mcal/kg of DM</td>
<td>3.0 ± 0.04</td>
<td>3.1</td>
<td>3.3</td>
<td>4.6 ± 0.03</td>
</tr>
</tbody>
</table>

\(^1\)Only 1 sample of each the combo and calf grower feeds were available and analysed.

\(^2\)Calf starter pellet (Mapleview HE Calf Starter) was supplied by Wallenstein Feed and Supply Ltd. (Wallenstein, Ontario, Canada), which included 52 mg/kg of monensin.

\(^3\)The calf grower ration (Mapleview Veal Starter 2.5:1) in the calf grower feed was supplied by Wallenstein Feed and Supply Ltd. (Wallenstein, Ontario, Canada), which included 22.3 mg/kg of monensin. The ingredients included were whole corn, Mapleview Veal Starter Supplement (MON/CHL), and soya oil mix.

\(^4\)Milk replacer was supplied by Mapleview Agri Ltd. (Palmerston, Ontario, Canada).

\(^5\)Calf starter pellet included ingredients: corn ground-500, soybean meal, soya hulls, wheat Ontario ground-650, alfalfa meal, DDGS-corn, lignin sulfonate (pellet binder), limestone B2, molasses liquid cond, sugar, soyplus, tallow in mix, salt feed mix WFS, dicalcium phosphate, micronutrient premix, vitamin ADE 8/1.25/8, magnesium oxide 56%, calf micronutrient premix, feed flavour, selisseo 0.1%, GLN cobalt 10 000 premix, zinc oxide 72%, and vitamin E 50% adsorbate.

\(^6\)Chopped straw was supplied by The Straw Boss Inc. (Mount Elgin, Ontario, Canada).

\(^7\)Chemical analysis was done by A&L Laboratory Services Inc (London, ON, Canada).

\(^8\)Fat for the rations is crude fat. Fat for the milk replacer was done by an acid hydrolysis test (AOAC, 1995: Method 954.02).

\(^9\)Lactose is assumed to be 100-CP-Fat-Ash.

\(^10\)Metabolizable Energy (ME) was calculated using NRC (2001) equations.
Table 2.2. Feeding program for all calves.

<table>
<thead>
<tr>
<th>Period</th>
<th>Week</th>
<th>Trial Days</th>
<th>Milk Replacer (Reconstituted with water; L/d)</th>
<th>Milk Replacer (Powder; g/d)</th>
<th>Ad-libitum Solid feed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk-fed</td>
<td>1</td>
<td>1-7</td>
<td>5</td>
<td>520</td>
<td>Calf starter</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8-14</td>
<td>5</td>
<td>650</td>
<td>Calf starter</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15-21</td>
<td>6</td>
<td>780</td>
<td>Calf starter</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>22-28</td>
<td>7</td>
<td>910</td>
<td>Calf starter</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>29-35</td>
<td>7</td>
<td>910</td>
<td>Calf starter</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>36-42</td>
<td>6</td>
<td>780</td>
<td>Calf starter</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>43-49</td>
<td>3</td>
<td>520</td>
<td>Calf starter</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>50-56</td>
<td>2.5</td>
<td>325</td>
<td>Calf starter</td>
</tr>
<tr>
<td>Post-wean</td>
<td>9</td>
<td>57-63</td>
<td>-</td>
<td>-</td>
<td>Combo + Straw (4%)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>64-70</td>
<td>-</td>
<td>-</td>
<td>Calf grower + Straw (4%)</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>71-77</td>
<td>-</td>
<td>-</td>
<td>Calf grower + Straw (4%)</td>
</tr>
</tbody>
</table>
Table 2.3. Analysis results (mean ± SD) of the *Echinacea purpurea* samples fed to calves on those treatments that received it.

<table>
<thead>
<tr>
<th>Component</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM (%)(^1)</td>
<td>97.3 ± 0.10</td>
</tr>
<tr>
<td>Aerobic plate count (CFU/g)</td>
<td>&lt;10.0 ± 0.00</td>
</tr>
<tr>
<td>Phenolics (ppm)</td>
<td></td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>&lt;60.0 ± 0.00</td>
</tr>
<tr>
<td>Cichoric acid</td>
<td>2332.5 ± 59.10</td>
</tr>
<tr>
<td>Echinacoside</td>
<td>&lt;60.0 ± 0.00</td>
</tr>
<tr>
<td>Caftaric acid</td>
<td>1587.5 ± 41.13</td>
</tr>
<tr>
<td>Total phenolics</td>
<td>3920.0 ± 100.00</td>
</tr>
</tbody>
</table>
Table 2.4. Multivariable linear regression model of the variables associated with blood haptoglobin concentration, segmented neutrophil count, lymphocyte count, and segmented neutrophils/lymphocytes ratio (N:L ratio).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Haptoglobin (g/L)</th>
<th>Segmented neutrophils (x10⁹/L)</th>
<th>Lymphocytes (x10⁹/L)</th>
<th>N:L ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>SE</td>
<td>P</td>
<td>Estimate</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.24</td>
<td>0.034</td>
<td>&lt;0.001</td>
<td>3.93</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON¹</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>E14²</td>
<td>-0.05</td>
<td>0.026</td>
<td>0.07</td>
<td>-0.41</td>
</tr>
<tr>
<td>E56³</td>
<td>-0.05</td>
<td>0.026</td>
<td>0.06</td>
<td>-0.57</td>
</tr>
<tr>
<td>Day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>14</td>
<td>-0.02</td>
<td>0.033</td>
<td>0.49</td>
<td>-1.15</td>
</tr>
<tr>
<td>28</td>
<td>0.01</td>
<td>0.033</td>
<td>0.84</td>
<td>0.10</td>
</tr>
<tr>
<td>57</td>
<td>0.09</td>
<td>0.034</td>
<td>0.01</td>
<td>-0.33</td>
</tr>
<tr>
<td>Room</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>2</td>
<td>0.04</td>
<td>0.026</td>
<td>0.12</td>
<td>0.23</td>
</tr>
<tr>
<td>3</td>
<td>0.03</td>
<td>0.027</td>
<td>0.29</td>
<td>0.68</td>
</tr>
<tr>
<td>Source</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auction</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Drover 1</td>
<td>-0.06</td>
<td>0.029</td>
<td>0.03</td>
<td>-</td>
</tr>
<tr>
<td>Drover 2</td>
<td>-0.03</td>
<td>0.024</td>
<td>0.23</td>
<td>-</td>
</tr>
</tbody>
</table>

¹CON = control, calves (n=39) received no *Echinacea purpurea*.
²E14 = calves (n=39) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28.
³E56 = calves (n=39) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56.
Table 2.5. Medication treatment protocol.

<table>
<thead>
<tr>
<th>Health score or symptom</th>
<th>Consecutive occurrence</th>
<th>Electrolytes (dose)</th>
<th>Medication treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces</td>
<td></td>
<td></td>
<td>Oral Meloxicam at 4.5 mL / 100 lbs (Diarrhea Treatment 1)</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>Oral Meloxicam at 4.5 mL / 100 lbs, and Borgal (Trimethoprim and Sulfadoxine) at 3 mL / 100 lbs delivered by subcutaneous (SQ) injection for 4 consecutive d (Diarrhea Treatment 2)</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1</td>
<td>Oral Meloxicam at 4.5 mL / 100 lbs, and Baytril (Enrofloxacin) at 4.5 mL / 100 lbs SQ (Diarrhea Treatment 3)</td>
</tr>
<tr>
<td>3 or 4 (with blood)</td>
<td>1</td>
<td>1</td>
<td>N/A</td>
</tr>
</tbody>
</table>
| Symptom                 |                        |                     | Treatment 1, 2 or 3  
| Refuses milk            | 1                      | 1                   | N/A |
| Refuses milk            | 3                      | 1                   | Treatment 1, 2 or 3  
| Appears dehydrated      | 1                      | 1                   | N/A |
| Too weak to stand       | 1                      | 1                   | Treatment 1, 2 or 3  
| Respiratory             |                        |                     | Follow protocol |
| 1<sup>st</sup> score ≥ 5| N/A                    | N/A                 | Nuflor (Florfenicol) at 6 mL / 100 lbs SQ, and 2 mL of Metacam (Meloxicam) SQ (Respiratory Treatment 1). |
| 2<sup>nd</sup> score ≥ 5| N/A                    | N/A                 | Excenel (Ceftiofur) at 2 mL / 100 lbs for 4 consecutive d delivered by intramuscular (IM) injection (Respiratory Treatment 2). |
| 3<sup>rd</sup> score ≥ 5| N/A                    | N/A                 | A-180 (Danofloxacin) at 2 mL / 100 lbs SQ, and 2 mL of Metacam SQ (Respiratory Treatment 3). |
| 4<sup>th</sup> score ≥ 5| N/A                    | N/A                 | Draxxin (Tulathromycin) at 1.1 mL / 100 lbs IM (Respiratory Treatment 4). |

<sup>1</sup> 1 dose of electrolytes = 2 L water and 115 g of Truvitalyte electrolyte powder (Mapleview Agri Ltd., Palmerston, Ontario, Canada). Electrolytes were fed 15 minutes after milk feedings.

<sup>2</sup> Dehydration symptoms: Sunken eyes, skin tents that take ≥ 5 seconds to return to flat, cold ears and nose, dull and depressed.
Table 2.6. Multivariable linear regression model of the variables associated with blood white blood cell, band neutrophil, monocyte, and basophil counts.

<table>
<thead>
<tr>
<th>Variable</th>
<th>White blood cells (x10⁹/L)</th>
<th>Band neutrophils (x10⁹/L)</th>
<th>Monocytes (x10⁹/L)</th>
<th>Basophils (x10⁹/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>SE</td>
<td>P</td>
<td>Estimate</td>
</tr>
<tr>
<td>Intercept</td>
<td>8.01</td>
<td>0.442</td>
<td>&lt;0.001</td>
<td>0.07</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON¹</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>E14²</td>
<td>-0.13</td>
<td>0.437</td>
<td>0.76</td>
<td>0.002</td>
</tr>
<tr>
<td>E56³</td>
<td>-0.32</td>
<td>0.439</td>
<td>0.47</td>
<td>-0.03</td>
</tr>
<tr>
<td>Day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>14</td>
<td>-0.42</td>
<td>0.306</td>
<td>0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>28</td>
<td>1.18</td>
<td>0.307</td>
<td>0.001</td>
<td>-0.02</td>
</tr>
<tr>
<td>57</td>
<td>1.38</td>
<td>0.309</td>
<td>&lt;0.001</td>
<td>-0.04</td>
</tr>
<tr>
<td>Room</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>2</td>
<td>0.30</td>
<td>0.438</td>
<td>0.50</td>
<td>-0.03</td>
</tr>
<tr>
<td>3</td>
<td>0.65</td>
<td>0.439</td>
<td>0.14</td>
<td>-0.02</td>
</tr>
<tr>
<td>FTPI⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

¹CON = control, calves (n=39) received no *Echinacea purpurea*.
²E14 = calves (n=39) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28.
³E56 = calves (n=39) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56.
⁴FTPI = failed transfer of passive immunity, defined as STP values < 5.2 g/dL.
⁵Monocyte data was log₁₀ transformed to convert the data to a normal distribution.
Table 2.7. Multivariable linear regression model of the variables associated with the blood IL-10, IL-6, and TNF-α concentrations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>IL-10 (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>TNF-α (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>SE</td>
<td>P</td>
</tr>
<tr>
<td>Intercept</td>
<td>2.32</td>
<td>0.025</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON1</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0.023</td>
<td>0.92</td>
</tr>
<tr>
<td>E142</td>
<td>-0.004</td>
<td>0.023</td>
<td>0.85</td>
</tr>
<tr>
<td>E563</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.14</td>
<td>0.023</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>28</td>
<td>0.24</td>
<td>0.023</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>57</td>
<td>0.18</td>
<td>0.023</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Room</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-0.05</td>
<td>0.023</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>-0.04</td>
<td>0.023</td>
<td>0.06</td>
</tr>
<tr>
<td>Source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auction</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Drover 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Drover 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FTPI4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1CON = control, calves (n=39) received no *Echinacea purpurea*.
2E14 = calves (n=39) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28.
3E56 = calves (n=39) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56.
4FTPI = failed transfer of passive immunity, defined as STP values < 5.2 g/dL.
5IL-10, IL-6, and TNF-α data was log_{10} transformed to convert the data to a normal distribution.
Table 2.8. Multivariable linear regression model of the variables associated with the proportion of fecal scores that were abnormal (scores 3 or 4; abnormal FS), proportion of fecal scores that were severe (score 4; severe FS), electrolyte doses, and the proportion of respiratory scores $\geq 4$ (RS $\geq 4$) in the milk-fed period.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Abnormal FS (%)$^5$</th>
<th>Severe FS (%)$^5$</th>
<th>Electrolyte doses (count)$^5$</th>
<th>RS $\geq 4$ (%)$^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>SE</td>
<td>$P$</td>
<td>Estimate</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.60</td>
<td>0.093</td>
<td>$&lt;0.001$</td>
<td>0.48</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON$^1$</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>E14$^2$</td>
<td>0.10</td>
<td>0.094</td>
<td>0.29</td>
<td>0.07</td>
</tr>
<tr>
<td>E56$^3$</td>
<td>-0.09</td>
<td>0.094</td>
<td>0.32</td>
<td>-0.03</td>
</tr>
<tr>
<td>Room</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>2</td>
<td>0.09</td>
<td>0.094</td>
<td>0.35</td>
<td>-0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.13</td>
<td>0.094</td>
<td>0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>Source</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auction</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>Drover 1</td>
<td>0.46</td>
<td>0.100</td>
<td>$&lt;0.001$</td>
<td>0.40</td>
</tr>
<tr>
<td>Drover 2</td>
<td>0.05</td>
<td>0.087</td>
<td>0.60</td>
<td>-0.02</td>
</tr>
<tr>
<td>FTPI$^4$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^1$CON = control, calves (n=80) received no *Echinacea purpurea*.
$^2$E14 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28.
$^3$E56 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56.
$^4$FTPI = failed transfer of passive immunity, defined as STP values $< 5.2$ g/dL.
$^5$Abnormal FS, severe FS, electrolyte doses and RS $\geq 4$ data was log$_{10}$ transformed to convert the data to a normal distribution.
Table 2.9. Cox proportional hazards model evaluating treatment for diarrhea with: 1) Oral Metacam (Meloxicam; Diarrhea Treatment 1) and with 2) Oral Metacam (Meloxicam) and Borgal (Trimethoprim and Sulfadoxine; Diarrhea Treatment 2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diarrhea Treatment 1</th>
<th></th>
<th>Diarrhea Treatment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard Ratio</td>
<td>95% Confidence interval</td>
<td>P</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON(^1)</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>E14(^2)</td>
<td>1.31</td>
<td>(0.86-1.99)</td>
<td>0.20</td>
<td>1.09</td>
</tr>
<tr>
<td>E56(^3)</td>
<td>0.93</td>
<td>(0.60-1.44)</td>
<td>0.73</td>
<td>0.96</td>
</tr>
<tr>
<td>Source</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auction</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>Drover 1</td>
<td>2.31</td>
<td>(1.52-3.51)</td>
<td>&lt; 0.001</td>
<td>2.31</td>
</tr>
<tr>
<td>Drover 2</td>
<td>1.08</td>
<td>(0.71-1.66)</td>
<td>0.71</td>
<td>0.85</td>
</tr>
<tr>
<td>Room</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>2</td>
<td>0.59</td>
<td>(0.38-0.91)</td>
<td>0.02</td>
<td>0.71</td>
</tr>
<tr>
<td>3</td>
<td>0.82</td>
<td>(0.54-1.23)</td>
<td>0.34</td>
<td>0.75</td>
</tr>
</tbody>
</table>

\(^1\)CON = control, calves (n=80) received no *Echinacea purpurea*.
\(^2\)E14 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28.
\(^3\)E56 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56.
<table>
<thead>
<tr>
<th>Variable</th>
<th>BRD risk</th>
<th>BRD risk</th>
<th>Odds ratio</th>
<th>Odds ratio</th>
<th>Mortality risk</th>
<th>Mortality risk</th>
<th>P</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.12 0.329</td>
<td>- -</td>
<td>0.72</td>
<td>Referent</td>
<td>- -</td>
<td>0.72</td>
<td>- -</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>CON&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Referent</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>E14&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-0.10 0.331</td>
<td>0.91 (0.472-1.74)</td>
<td>0.77</td>
<td>Referent</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>E56&lt;sup&gt;3&lt;/sup&gt;</td>
<td>-0.29 0.330</td>
<td>0.75 (0.392-1.436)</td>
<td>0.38</td>
<td>Referent</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>Room 1</td>
<td>Referent</td>
<td>- -</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>2</td>
<td>-0.62 0.339</td>
<td>0.54 (0.275-1.048)</td>
<td>0.07</td>
<td>Referent</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>3</td>
<td>0.16 0.328</td>
<td>1.18 (0.616-2.248)</td>
<td>0.62</td>
<td>Referent</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>Source</td>
<td>Auction Referent</td>
<td>- -</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>Drover 1</td>
<td>0.45 0.356</td>
<td>1.57 (0.779-3.169)</td>
<td>0.21</td>
<td>Referent</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>Drover 2</td>
<td>-0.38 0.305</td>
<td>0.68 (0.375-1.249)</td>
<td>0.22</td>
<td>Referent</td>
<td>- -</td>
<td>Referent</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>FTPI&lt;sup&gt;4&lt;/sup&gt;</td>
<td>No</td>
<td>Referent</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>Yes</td>
<td>0.84 0.301</td>
<td>2.32 (1.279-4.19)</td>
<td>0.01</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
</tbody>
</table>

<sup>1</sup>CON = control, calves (n=80) received no *Echinacea purpurea*.
<sup>2</sup>E14 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28.
<sup>3</sup>E56 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56.
<sup>4</sup>FTPI = failed transfer of passive immunity, defined as STP values < 5.2 g/dL.
<sup>5</sup>BRD risk data was created by assigning a 1 to calves that had at least 1 respiratory score ≥5, and a 0 to calves that had no respiratory scores ≥5.
<sup>6</sup>Mortality risk data was created by assigning a 1 to calves that died, and a 0 to calves that survived.
Table 2.11. Multivariable linear regression model of the variables associated with milk replacer (MR) intake, grain intake, and feed conversion rate (FCR) in the milk-fed period.

<table>
<thead>
<tr>
<th>Variable</th>
<th>MR intake (kg)</th>
<th>Grain intake (kg)</th>
<th>FCR (Mcal ME intake/kg BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>SE</td>
<td>P</td>
</tr>
<tr>
<td>Intercept</td>
<td>34.04</td>
<td>0.956</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON(^1)</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E14(^2)</td>
<td>1.55</td>
<td>0.961</td>
<td>0.11</td>
</tr>
<tr>
<td>E56(^3)</td>
<td>0.30</td>
<td>0.960</td>
<td>0.76</td>
</tr>
<tr>
<td>Week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Room</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-0.45</td>
<td>0.960</td>
<td>0.64</td>
</tr>
<tr>
<td>3</td>
<td>-1.04</td>
<td>0.960</td>
<td>0.28</td>
</tr>
<tr>
<td>Source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auction</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Drover 1</td>
<td>-2.48</td>
<td>1.027</td>
<td>0.02</td>
</tr>
<tr>
<td>Drover 2</td>
<td>0.33</td>
<td>0.891</td>
<td>0.71</td>
</tr>
</tbody>
</table>

\(^1\)CON = control, calves (n=80) received no *Echinacea purpurea*.  
\(^2\)E14 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28.  
\(^3\)E56 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56.  
\(^4\)MR powder, DM basis.  
\(^5\)Calf starter, DM basis, calculated at the pod level.  
\(^6\)FCR was calculated based on metabolizable energy (ME) intake, at the pod level.
Table 2.12. Multivariable linear regression model of the variables associated with calf hip height, weekly BW, and average daily gain (ADG) in the milk-fed period.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hip height (cm)</th>
<th>Weekly BW (kg)</th>
<th>ADG (kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>SE</td>
<td>P</td>
</tr>
<tr>
<td>Intercept</td>
<td>64.96</td>
<td>2.031</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E14&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.29</td>
<td>0.339</td>
<td>0.39</td>
</tr>
<tr>
<td>E56&lt;sup&gt;3&lt;/sup&gt;</td>
<td>-0.06</td>
<td>0.340</td>
<td>0.86</td>
</tr>
<tr>
<td>Day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>1.48</td>
<td>0.171</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>28</td>
<td>4.27</td>
<td>0.161</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>57</td>
<td>9.85</td>
<td>0.162</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Room</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.42</td>
<td>0.342</td>
<td>0.22</td>
</tr>
<tr>
<td>3</td>
<td>0.62</td>
<td>0.344</td>
<td>0.07</td>
</tr>
<tr>
<td>FTFPI&lt;sup&gt;4&lt;/sup&gt;</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arrival BW</td>
<td>0.43</td>
<td>0.041</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>1</sup>CON = control, calves (n=80) received no Echinacea purpurea.

<sup>2</sup>E14 = calves (n=80) received 3 g/d of Echinacea purpurea split over 2 milk feedings from d 14-28.

<sup>3</sup>E56 = calves (n=80) received 3 g/d of Echinacea purpurea split over 2 milk feedings from d 1-56.

<sup>4</sup>FTPI = failed transfer of passive immunity, defined as STP values < 5.2 g/dL.

<sup>5</sup>Body weights were taken at the end of each listed week (Eg. Week 1’s body weight was taken on day 7).
Table 2.13. Multivariable linear regression model of the variables associated with the proportion of respiratory scores with no symptoms of bovine respiratory disease (RS = 0), grain intake, average daily gain (ADG), and feed conversion rate (FCR) in the post-wean period.

<table>
<thead>
<tr>
<th>Variable</th>
<th>RS = 0 (%)</th>
<th>Grain intake (kg)&lt;sup&gt;5&lt;/sup&gt;</th>
<th>ADG (kg/d)</th>
<th>FCR (Mcal ME intake/kg BW)&lt;sup&gt;6&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>SE</td>
<td>P</td>
<td>Estimate</td>
</tr>
<tr>
<td>Intercept</td>
<td>86.96</td>
<td>2.540</td>
<td>&lt;0.001</td>
<td>66.91</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>E14&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-4.02</td>
<td>2.866</td>
<td>0.16</td>
<td>-0.52</td>
</tr>
<tr>
<td>E56&lt;sup&gt;3&lt;/sup&gt;</td>
<td>-0.84</td>
<td>2.906</td>
<td>0.77</td>
<td>5.58</td>
</tr>
<tr>
<td>Week</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>18.00</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>47.21</td>
</tr>
<tr>
<td>Room</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>2</td>
<td>-1.22</td>
<td>2.873</td>
<td>0.67</td>
<td>16.36</td>
</tr>
<tr>
<td>3</td>
<td>8.90</td>
<td>2.887</td>
<td>0.002</td>
<td>14.15</td>
</tr>
</tbody>
</table>

<sup>1</sup>CON = control, calves (n=80) received no *Echinacea purpurea*.

<sup>2</sup>E14 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28.

<sup>3</sup>E56 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56.

<sup>5</sup>Combo or calf grower feed + straw, DM basis, calculated at the pod level.

<sup>6</sup>FCR was calculated based on metabolizable energy (ME) intake, at the pod level.
### Table 2.14. Multivariable logistic regression model of the variables associated with the risk of bovine respiratory disease (BRD) and the risk of mortality in the post-wean period.

<table>
<thead>
<tr>
<th>Variable</th>
<th>BRD risk</th>
<th>Odds ratio</th>
<th>Mortality risk</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>SE</td>
<td>95% Confidence</td>
<td>Estimate</td>
</tr>
<tr>
<td>Intercept</td>
<td>-1.55</td>
<td>0.429</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON(^1)</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>E14(^2)</td>
<td>-0.11</td>
<td>0.560</td>
<td>0.90</td>
<td>(0.298-2.703)</td>
</tr>
<tr>
<td>E56(^3)</td>
<td>-0.61</td>
<td>0.648</td>
<td>0.54</td>
<td>(0.151-1.946)</td>
</tr>
<tr>
<td>Room</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
<td>Referent</td>
</tr>
<tr>
<td>2</td>
<td>-0.52</td>
<td>0.518</td>
<td>0.60</td>
<td>(0.215-1.655)</td>
</tr>
<tr>
<td>3</td>
<td>-2.50</td>
<td>1.060</td>
<td>0.08</td>
<td>(0.01-0.661)</td>
</tr>
</tbody>
</table>

\(^1\)CON = control, calves (n=80) received no *Echinacea purpurea*.

\(^2\)E14 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28.

\(^3\)E56 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56.

\(^4\)BRD risk data was created by assigning a 1 to calves that had at least 1 respiratory score \(\geq 5\), and a 0 to calves that had no respiratory scores \(\geq 5\).

\(^5\)Mortality risk data was created by assigning a 1 to calves that died, and a 0 to calves that survived.
Table 2.15. Cox proportional hazards model evaluating treatment for respiratory disease with Nuflor (Florfenicol) and Metacam SQ (Meloxicam; Respiratory Treatment 1), including the milk-fed and post-wean periods.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>95% Confidence interval</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON$^1$</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E14$^2$</td>
<td>0.99</td>
<td>(0.65-1.52)</td>
<td>0.98</td>
</tr>
<tr>
<td>E56$^3$</td>
<td>0.87</td>
<td>(0.56-1.35)</td>
<td>0.54</td>
</tr>
<tr>
<td>Room</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.59</td>
<td>(0.38-0.93)</td>
<td>0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.98</td>
<td>(0.65-1.48)</td>
<td>0.92</td>
</tr>
<tr>
<td>FTPI$^4$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Referent</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>1.81</td>
<td>(1.26-2.61)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

$^1$CON = control, calves (n=80) received no *Echinacea purpurea*.
$^2$E14 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28.
$^3$E56 = calves (n=80) received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56.
$^4$FTPI = failed transfer of passive immunity, defined as STP values < 5.2 g/dL.
Figure 2.1. Eosinophils by treatment and arrival BW. Each dot represents a calf. The treatments included: CON = control (calves received no *Echinacea purpurea*), E14 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28, and E56 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56. N=39 calves/treatment.
Figure 2.2. Rectal temperature (back-transformed mean +/- 95% CI) by d (a = 14, b = 28, and c = 57), treatment and FTPI status. FTPI = failed transfer of passive immunity, defined as STP values < 5.2 g/dL. Within FTPI status (no or yes), significant differences (P ≤ 0.05) detected between treatments are denoted by ‘*’, and tendencies for differences (0.05 < P < 0.1) are denoted by ‘†’. The treatments included: CON = control (calves received no *Echinacea purpurea*), E14 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28, and E56 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56. N=39 calves/treatment.
**Figure 2.3.** Proportion (mean +/- SE) of respiratory scores with no symptoms of bovine respiratory disease (RS = 0) in the milk-fed period by treatment and source. Within each source, significant differences (P ≤ 0.05) detected between treatments are denoted by ‘*’, and tendencies for differences (0.05 < P ≤ 0.1) are denoted by ‘†’. The treatments included: CON = control (calves received no *Echinacea purpurea*), E14 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28, and E56 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56. N=80 calves/treatment.
Figure 2.4. Proportion (back-transformed mean +/- 95% CI) of respiratory scores ≥ 4 (RS ≥ 4) in the post-wean period by treatment and source. Within each source, significant differences (P ≤ 0.05) detected between treatments are denoted by ‘*’, and tendencies for differences (0.05 < P ≤ 0.1) are denoted by ‘†’. The treatments included: CON = control (calves received no *Echinacea purpurea*), E14 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28, and E56 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56. N=80 calves/treatment.
Figure 2.5. Average post-wean period weekly body weight by treatment and arrival body weight. Each dot represents a calf. The treatments included: CON = control (calves received no *Echinacea purpurea*), E14 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 14-28, and E56 = calves received 3 g/d of *Echinacea purpurea* split over 2 milk feedings from d 1-56. N=80 calves/treatment.
Figure 2.6. Diagram of the room: milk-fed period layout on the left, individual stalls were 101.6 cm tall (A), 78.74 cm wide (B), 121.92 cm long (C), and the post-weaning period pens (pods) on the right were the same height, 198.12 cm wide (D) and 396.24 cm long (E).
3 CHAPTER 3: EFFECTS OF WEANING AND TYNDALLIZED
*LACTOBACILLUS HELVETICUS* SUPPLEMENTATION ON DAIRY
CALF BEHAVIORAL AND PHYSIOLOGICAL INDICATORS OF
AFFECTIVE STATE

3.1 INTRODUCTION

Weaning, the process of transitioning dairy calves from a diet containing milk to one
entirely composed of solid feed, is a necessary, but potentially negative, experience for dairy
calves (Jasper et al., 2008). There are many reasons why weaning may impact dairy calf affective
state. Milk is often fed through a nipple, which facilitates sucking behavior - a behavior calves
are motivated to perform (de Passille, 2001), and one that may elicit satiety (de Passille et al.,
1993) and calming effects (Veissier et al., 2002). Weaning removes these experiences that may
contribute to a positive affective state. Calves prefer to drink milk (Webb et al., 2014) and it is
their natural source of nutrition in early in life (Khan et al., 2016), therefore, removing milk
could inflict negative affect. Additionally, there is high variability among calves regarding when
they begin to eat solid feed and how quickly their consumption increases during weaning (de
Passille and Rushen, 2016). Yet abrupt weaning methods still occur (Vasseur et al., 2010), which
can result in growth depressions (Steele et al., 2017). In addition, on most dairy farms in North
America, calves are weaned much earlier (6-8 wk of age; Vasseur et al., 2010) than they would
be if raised by their dam in a natural environment (average 10 mo; Reinhardt and Reinhardt,
Calves weaned early show more signs of hunger (negative affect) than calves weaned later and/or by a specified amount of voluntary starter intake (hunger inferred by more unrewarded visits to an automated milk feeder; de Passille and Rushen, 2016).

Another factor that may influence affective state at weaning is the gut microbiota, or microorganisms in the gut (Cryan and Dinan, 2012). Gut microbiota are not only essential to gut and overall animal health and function (Malmuthuge and Guan, 2017), they also play a major role in mental well-being through their key involvement in the gut-brain axis (Huang and Wu, 2021). The gut-brain axis is the bi-directional communication pathway between the gut and the brain, involving the nervous, endocrine, and immune systems (Makris et al., 2021), which links emotional and cognitive brain regions with gut function (Jenkins et al., 2016). Undesirable alterations in gut microbiota composition and/or diversity, termed “dysbiosis,” and psychological stress are linked (Cryan and Dinan, 2012). Correspondingly, gut microbiota are reported to change at weaning. For example, Meale et al. (2017) reported that the most abundant ruminal phyla in dairy calves changed from Bacteroidetes to Firmicutes at weaning, the change was more rapid in calves weaned at 6 weeks as opposed to 8 weeks. Similarly, Li et al. (2018) reported weaning decreased some beneficial species, including Alloprevotella and Oscillibacter, and increased some pathogenic species, including Campylobacterales, Campylobacteraceae, and Campylobacter, in piglets. Factors likely to affect this are the change in diet (Meale et al., 2016) and psychological stress (de Palma et al., 2014). Therefore, methods to support the gut microbiota and the gut-brain axis may prevent or reduce negative affects associated with
weaning. Probiotics and postbiotics have potential and warrant research to support dairy calf welfare.

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO and WHO, 2002). Probiotics have been supplemented to support gut and overall physical health in many species, including dairy calves (Cangiano et al., 2020). Probiotics may also benefit mental health through their modulation of the gut-brain axis (Zhang et al., 2020), positively affecting brain physiology and behavior (Cryan and Dinan, 2012). While probiotics have received the majority of focus in the literature to date, dead probiotics, most often killed by heat (Adams, 2010), also referred to in the literature as “tyndallized probiotics”, “ghost probiotics”, “paraprobiotics”, and “postbiotics”; de Almada et al., 2016; Piqué et al., 2019) have gained increased attention since they can yield similar beneficial health affects while being safer, in that they will not cause infection, and may be easier to transport and store (Adams, 2010; Piqué et al., 2019). The term “postbiotic” has been defined as: “non-viable bacterial products or metabolic byproducts from probiotic microorganisms that have biologic activity in the host” (de Almada et al., 2016). More research is needed on probiotics, and especially on postbiotics, as the mechanisms of these are not well understood (Deshpande et al., 2018; Piqué et al., 2019; Cuevas-González et al., 2020). It is known that postbiotics function differently than probiotics in that they are dead and cannot colonize in the gut (Meng et al., 2022). Despite this, probiotics and postbiotics may share some mechanisms and effects (Adams, 2010). Part of the reason for this could be because probiotic products will
inevitably include a portion of dead microbes due to lost viability over time (Nighswonger et al., 1996) and, therefore, effects from such products may be representative of not only the live microorganisms, but the dead as well. Overall, postbiotics contain non-viable microbial cells (including their structural components) and a wide range of metabolic by-products from live probiotic bacteria that can exert beneficial biological effects on the gut-brain axis through nervous, endocrine, and immune routes just like probiotics (Nataraj et al., 2020; Noh et al., 2022). These bacterial components can exert anti-inflammatory, immunomodulatory, antiproliferative, antioxidant, and antimicrobial bioactivities (Taverniti and Guglielmetti, 2011; Dahiya and Nigam, 2022). Furthermore, heat treatment may inflict unique beneficial effects compared to probiotics, as it can rupture cell walls which release the cell contents, making them more available to the host, facilitating the success of their beneficial effects (Piqué et al., 2019). Postbiotics have been reported in some studies to cause beneficial changes in the gut microbiota composition: Canani et al. (2017) reported supplementation of heat-killed Lactobacillus paracasei CBAL74 promoted the development of butyrate producing microbes which was associated with improved immunity in children. Postbiotics can also antagonize pathogens: Liu et al. (2017) reported exopolysaccharide, extracted from Lactobacillus plantarum WLPL04 inhibited adhesion of pathogens and their biofilm production. Postbiotics may alter levels of hormones/neurotransmitters: Hara et al. (2018) reported mice supplemented with heat-killed Lactobacillus casei subsp. casei 327, had higher serotonin levels in colon tissue compared to control mice. These are effects that would support gut and overall animal health, and likely extend to mental well-being through the gut-brain axis. There is little research available on how
probiotics impact affective state in cattle (Kelsey et al., 2018), although of the studies that assessed this, although few measures were assessed, most reported results that may be indicative of reduced negative affect (Zhang et al., 2016; Kelsey et al., 2018; Lee et al., 2019; Xie et al., 2020). While there were no studies identified in the literature that investigated the effects of postbiotics on cattle affective state.

Additionally, the mechanisms and benefits of probiotics and postbiotics can be genre, species, and/or strain specific (de Almada et al., 2016; Cuevas-González et al., 2020). Due to limited research identified on *Lactobacillus helveticus* (LH) in relation to mental well-being, all study results, regardless of strain, are shared here. Research in rodents and humans supports that LH can improve stress-related symptoms. Considering probiotic research, Liang et al. (2015) reported supplementation of LH to stressed rats improved behavioral (anxiety, depression and cognitive) dysfunction better than an anti-depressant (selective serotonin reuptake inhibitor-citalopram). Also, LH was associated with lower plasma corticosterone and adrenocorticotropic hormone levels, higher plasma interleukin-10 levels, restored hippocampal serotonin and norepinephrine levels, and more hippocampal brain-derived neurotrophic factor mRNA expression. Likewise, LH supplementation was associated with reduced anxiety-like behavior in mice (Ohland et al., 2013) and improved learning and memory in mice (Ohsawa et al., 2015). *Lactobacillus helveticus* R0052 supplementation in addition to *Bifidobacterium longum* R0175 was associated with reduced anxiety-like behavior in rats and psychological distress in humans (Messaoudi et al., 2011). Considering postbiotic research, Maehata et al. (2019) reported that
supplementation of tyndallized LH (TLH) to stressed mice improved anxiety- or depressive-like behaviors and stress-induced gene expression alterations. While they did not detect any effect of TLH supplementation on the gut microbiota composition, they suggested the beneficial effects could be attributable to vagus nerve stimulation, altering the metabolites produced by the gut microbiota, and/or modulating the immune system (cytokines), although stated deeper investigation is required to identify and elucidate the mechanisms. No previous studies were identified that assessed the effect of live LH or TLH on measures of cattle (including dairy calves) affective state.

Therefore, the objectives of this study were to determine if weaning would induce physiological and behavioral indicators of negative affective state and if supplementation of TLH to dairy calves would result in reduced indicators of negative affect during weaning. The hypothesis was that weaning would induce behavioral and physiological indicators of negative affective state and that those indicators would be reduced in calves receiving TLH.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Housing

This study was part of a larger trial (Cangiano et al., in preparation) focused on investigating the effects of TLH supplementation on calf immunity, gut health, intake, and growth. A randomized trial was conducted at a University of Guelph animal research facility (Ponsonby General Animal Facility, Ponsonby, Ontario, Canada) using 2 batches of calves
(batch 1 and 2 included 13 and 10 calves, respectively), for a total sample size of 23 singlet male Holstein calves. All calves were sourced from another University of Guelph animal research facility (Ontario Dairy Research Centre at the Elora Research Station, Elora, Ontario, Canada) located less than 10 km away. All study procedures were reviewed and approved by the University of Guelph Animal Care Committee (AUP#4470).

As soon as possible after birth, farm staff transferred calves from an individual maternity pen with their dam to a pre-disinfected and washed calf crate. Calves had their navels thoroughly dipped in iodine and were fed their first colostrum meal promptly (described below). All calves were healthy at birth based on overall appearance, rectal temperature, umbilical appearance, and nasal discharge. All calves were transported to the Ponsonby General Animal Facility and enrolled in the study on d 1 of life. The calf crates were transported in the back of a truck from one research facility to the other. The calf crates were disinfected with Virkon (CDMV, Brampton, Ontario, Canada) and pressure washed between calves. For each batch, calves were enrolled over approximately 1-2 mo. (calves in batch 1 were enrolled between July 6, 2021 to August 17, 2021, and calves in batch 2 were enrolled between September 6, 2021 and November 2, 2021). There was 1 calf (not included in the 23 calf total sample size) removed from the study within the first 21 d of age due to an injury.

There were 4 rooms at the facility: rooms 1-3 were structurally identical while room 4 was smaller. Rooms 1-3 had 2 rows of pens (9 pens total), with the rows separated by a walkway, while room 4 had 1 row of 4 pens with a walkway in front. All pens were 1.20 x 1.80 x
3.60 m (height x width x length). The pens were vertical metal bar-sided (1.50 cm width, 6.00 cm apart), except 1-2 sides which were solid concrete walls depending on the pen’s location in the room. The pens had concrete floors, and were bedded with wood shavings, with a straw pack in the back half. On the inside of each pen, there were 2 bucket holders for solid feed and water buckets, as well as a hook for the milk bucket. After milk feedings, each calf’s milk bucket was thoroughly scrubbed using a bristled scrub brush, hot water and Liquid Hand Soap (Agrisan, Arthur, Ontario, Canada), and then rinsed with water. The attached nipples were also cleaned by squeezing the hot soapy water through them and then squeezing clean water through them to rinse. Each calf’s bucket was then hung outside of their pen.

For each batch of calves, rooms were filled 1 at a time to keep calves of similar ages together. Within each room, a pen was kept empty between calves on different treatments to prevent microbiota transfer. At any given time, a maximum of 6 calves were housed in each of rooms 1-3, while a maximum of 3 calves were housed in room 4. Calves had auditory and visual contact with other calves, however, physical contact was limited due to the metal bar pen sides. Shavings were cleaned out of each pen and replaced daily, while straw was topped up throughout the wk as needed, and the whole pen was completely cleaned out and re-bedded 1/wk. As calves in batch 1 completed the trial, they were removed from their pens, which were disinfected with MS MegaDes Novo (Schippers Canada Ltd., Lacombe, Alberta, Canada), pressure washed, and prepared for the next calf (in batch 2). Calf rooms were artificially lit by ceiling lights and the room temperature was set at 12°C. The study began in the summer and finished in the winter,
resulting in variable outdoor temperatures. When the room temperatures began to rise over 12°C, the exhaust fans ramped up to maximize fresh air coming into the rooms and when the room temperatures began to drop under 12°C, the overhead heaters would turn on. Calves remained on the study until d 42.

### 3.2.2 Feeding and Health Management

All calves were fed 2 meals of a 26% IgG standardized colostrum replacer (CR) powder (HeadStart, Saskatoon Colostrum Company Ltd., Saskatoon, Saskatchewan, Canada) on the first day of life - the first within 6 h after birth while still at the Elora Research Station and the second at approximately 12 h after birth, when they were at the Ponsonby General Animal Facility. Each colostrum meal consisted of 750 g of CR powder mixed with water between 42-45oC to reach a final volume of 3 L to deliver 200 g of IgG to the calf. All CR, milk replacer (MR), and electrolytes were prepared in water at this temperature. Both colostrum meals were prepared in a pail and fed in a calf bottle fitted with a nipple. The calves were encouraged to drink from the bottle, however, if they did not consume all of the colostrum meals, the refusals were fed with an esophageal tube feeder. During the second day of life, the calves were fed 2 feedings of a 50:50 mix of CR and MR (Grober Nutrition, Cambridge, Ontario, Canada; Table 3.1), each meal consisting of 225 g of CR powder and 225 g of MR powder mixed with water to reach a final volume of 3 L. The CR:MR meals were prepared in a pail and fed in a bucket fitted with a nipple. A 26% crude protein (CP), and 20% fat MR powder was used. The MR contained no feed additives, including no antimicrobials. Animal handlers remained with the calves during
these feedings and trained the calves to consume milk from the buckets fitted with nipples by allowing the calves to suckle on their fingers and guiding their mouth onto the nipple. At approximately 24 h after the first colostrum meal, a blood sample was taken and a Brix measurement obtained using a digital brix refractometer (Digital Refractometer, Misco, Solon, Ohio, United States) to infer about transfer of passive immunity status. The average level of Brix % was $9.1 \pm 0.42\%$ (min = 8.4%, max = 10.2%) and based on Lombard et al. (2020), all calves had fair to excellent passive immunity. There was no difference detected between the treatments.

Calves had ad libitum access to fresh water in a bucket from d 1 of life. Starting on d 3 of life, calves received 6 L/d of MR, which was increased to 9 L/d on d 7 of life. Daily MR allowance was fed split over 3 meals (0800, 1400, and 2000 h) at 150 g of MR powder/L. The MR was prepared with water either by hand in a pail or in a milk taxi (MilkTaxi100, Holm & Laue, Westerrönfeld, Germany) depending on the number of calves at the facility at the time, and fed to calves in the same buckets fitted with nipples. All colostrum, milk, and electrolyte feedings were conducted by farm staff (3 people) or researchers and volunteers (up to 10 people).

Calves were offered a high-starch texturized starter (40% starch, and 20% Neutral Detergent Fiber; NDF; Shur-Gain, Ontario, Canada; Table 3.1) on d 28 and fed ad libitum for the rest of the study. The calf starter contained no feed additives, including no antimicrobials. Orts were discarded and starter was replaced or toped up fresh as needed. Calves were weaned from MR with a stepdown method as follows: on d 35 the MR was reduced to 6 L/d (2 L /meal), on d
37 the MR was reduced to 3 L/d (1 L/meal), and on d 39 the MR was reduced to 0.4 L/d (0.2 L/meal, fed at 0800 and 2000 h meals only), which the calves received until d 42.

Health scoring was performed to monitor health and detect any illness quickly, conducted daily starting at 1000 h by 1 of 3 trained researchers using the Wisconsin Calf Health Scoring App (University of Wisconsin-Madison School of Veterinary Medicine, Madison, Wisconsin, United States). The app allowed nose, eye, ear, appetite, attitude, cough, temperature, fecal, navel, and joint scores to be recorded, with possible scores 0-3 for each. All scores were recorded directly into the app, and then data were transferred to a Microsoft Excel (Microsoft Corp., Redmond, Washington, United States) spreadsheet. A fecal score of 2 or 3 was considered diarrhea (McGuirk et al., 2008). At the first occurrence of diarrhea, calves were administered meloxicam (Metacam; Boehringer Ingelheim, Ingelheim, Germany) at 1.25 ml/50 kg subcutaneously and were offered electrolytes 1 - 2 h following the 0800 and 1400 h feedings for two consecutive d or until the fecal score was normal. Each electrolyte feeding consisted of 76 g of oral electrolytes (Calf-Lyte: 112.5 mEq/L Na, 43 mEq/L Cl, 15 mEq/L K, 10.5 mEq/L PO4, Vetoquinol, Lavaltrie, Québec, Canada) diluted in 2.25 L of water and fed to calves in a bottle or bucket fitted with a nipple. If a calf completely refused to drink the electrolytes, still had diarrhea, and were showing signs of dehydration, they were tubed the electrolytes via an esophageal tube feeder. For calves with diarrhea and other severe clinical signs of illness (very dull, depressed, and weak with sunken eyes), a veterinarian was consulted to decide on a
treatment plan. Respiratory illness was treated on an individual calf basis through consultation with a veterinarian. No calves were treated with antimicrobials during the trial.

Calves were vaccinated with a modified live virus oral vaccine (Bovilis Nasalgen-3PMH) on d 7 of life against Bovine Rhinotracheitis virus, Bovine Respiratory Syncytial Virus, Parainfluenza 3 virus, *Mannheimia haemolytica*, and *Pasteurella multocida*. Animal handlers put on a clean pair of gloves when moving between calves. They also put on a new pair of plastic boot covers between calf rooms and scrubbed their boot covers in prepared disinfectant solution (Virkon, CDMV, Brampton, Ontario, Canada) between calves. These measures were taken to prevent microbial spread between calves.

### 3.2.3 Treatments Allocation

Treatments were allocated to pens in each room prior to calves arriving. The treatments included: 1) control (CON), no TLH (n = 12), and 2) supplementation of tyndallized *Lactobacillus helveticus* R0052 (TLH), 5 g/d of TLH at $10^9$ CFU/g (n = 11). The sample size for the study was based on the primary outcomes of the larger study, including IL-6 concentration (Cangiano et al., in preparation). External personnel assigned letters (e.g. A and B) to the treatments, to allow the primary author and fellow researchers to be blinded to the treatments. The CON treatment consisted of the same carrier ingredient (lactose) as the postbiotic treatment, which allowed both treatments to visually appear identical. Treatments were administered from d 3-42. Each day, 2.5 g of treatment/calf was fed at each of the 0800 and 2000 h feedings, mixed
individually into each calf’s bucket with 1 L of milk to ensure they consumed all the product, and then the rest of the milk allowance was provided. During the last phase of weaning, the treatments were mixed into the 0.2 L of milk offered twice daily to keep the method of postbiotic administration consistent.

3.2.4 Measurements and Sample Collection

Behavioral measurements collected to infer about the calves’ affective state included lying behavior, play behavior, and a cognitive task. Physiological measurements collected to infer about the calves’ affective state included saliva cortisol, maximum eye temperature (MET), and blood serotonin.

Electronic data loggers (HOBO Pendant G Data Logger, Onset Computer Corp, Bourne, Massachusetts, United States), validated by Bonk et al. (2013), were programmed to record leg orientation every 60 s, wrapped in veterinary bandaging tape, and secured horizontally onto each calf’s inner-rear right leg with veterinary bandaging tape (3M Vetrap Bandaging Tape, London, Ontario, Canada) on d 21. On d 28, the logger was removed and replaced with a new logger promptly secured in the same manner although to the opposite rear leg. The same procedure was repeated again on d 35 and then removed on d 42. The time of d and date each time a HOBO was placed on and/or taken off a calf was recorded for later HOBO data analysis. Each time a HOBO logger was removed, data were downloaded onto a computer using HOBOware Pro Software (Onset Computer Corp; Bourne, Massachusetts, United States) and imported into Microsoft
Excel (Microsoft Corp; Redmond, Washington, United States). The data were then analyzed to determine lying bout frequency (bouts/d), lying duration (min/d), and daily average lying bout length (min/bout), and for each calf (Overvest et al., 2018; Reedman et al., 2021).

Play behavior was induced by bedding the calves with shavings, as done by Reedman et al. (2021). Each calf was tested in this manor around 0900 h on d 33, 37, and 41, in their own pen. On these days, the bedding to induce play behavior replaced that morning’s shavings provision. At the time of testing, a video camera (GoPro Hero 9, GoPro, California, United States) was attached to a tri-pod and positioned in front of the calf pen to capture the entire calf pen, and the video recording started. The researcher got the calf up if not already standing, and then using a shovel, quickly (within 1 min) bed the front half of the calf’s pen with 8 shovelfuls of clean wood shavings. All shavings were tossed low to the ground to avoid tossing shavings on the calf and prevent the room from getting dusty. Once the researcher finished bedding, they left the room for 3 min so there were no people in the room during the play assessment. Time spent playing and a differentiation of various calf play behaviors based on an ethogram (Table 3.2) was later determined using the behavior coding program, Solomon Coder (Peter, 2019). For each play assessment, behavior was coded from 30 sec before the gate was closed (bedding completed) to 180 sec after the gate was closed (a total of 210 sec). Two people coded all play videos. Inter-observer reliability was calculated for each behavior by the Kappa coefficient (k), including 20% of the videos. The average Kappa (κ) was 86%. κ > 0.76.
Each calf was tested to perform a detour task on d 38 and 39 between 1000 h and 1300 h. A see-through, V-shaped apparatus was centered between the left and right sides of a 3.66 m wide by 7.32 m long pen bedded with shavings, with the point of the V 3 m away from the middle of the front of the pen, with the sides (positioned approximately 30 degrees apart) extending towards the back of the pen (Figure 3.1). The apparatus was made of 2, 3.5 m long, 1.1 m tall aluminum gates, attached using a metal bar and zip ties at the point where the gates met to create the V shape (detour). An empty milk bucket (identical to the ones the calves received their milk meals in) was hung using zip ties inside the point of the V matching the calves’ normal drinking height with the nipple facing outwards to where the V opens up. A small amount of MR (< 0.4 L) was prepared and poured into the bucket. See Figure 3.1 for a visual of the detour apparatus and pen layout, which was built based off of that described by Nawroth et al. (2016) and personal communication with Dr. Heather Neave (Post-doctoral Research Fellow, Aarhus University) and Laura Whalin (PhD student, University of British Columbia). A video camera (GoPro Hero 9, GoPro, California, United States) was attached to the tripod and secured approximately 4 m above the ground of the pen, positioned near the center to capture the entire pen. The video recording was started, the calf was guided from their home pen directly to the testing pen, once all 4 hooves were on the ground inside the testing pen, the test began. The test ended when the calf found their way around the apparatus and touched the bucket/nipple or when 5 min passed, whichever came first. Later, the test time duration was determined from watching the video recordings. The pen (including the detour apparatus) was disinfected with Virkon (CDMV, Brampton, Ontario, Canada), pressure-washed, and re-bedded between calves.
Saliva sampling was done on d 33, 37, and 41 at approximately 0930 h. A researcher entered the calf’s pen and held the calf gently, but securely, and offered the calf a swab (Salivabio Children’s Swab, Salimetrics LLC, Pennsylvania, United States). If the calf did not grab onto the swab, the researcher slowly inserted the swab into the calf’s mouth, held loosely with a sterile glove in the calf’s mouth, until the bottom 2/3 was well soaked with saliva (at least 1 mL; Loberg et al., 2008; Kovács et al., 2021) and then the swab was placed into a sterile plastic tube (Swab Storage Tube, Salimetrics LLC, Pennsylvania, United States). Two swabs were collected/calf on each sample d, 1 to be analyzed and 1 to be kept as a spare. This method of saliva collection appeared to cause little stress and be well accepted by the calves. The tubes were stored at −20°C until analysis (Meléndez et al., 2018). Samples were sent to Salimetrics (Carlsbad, California, United States) where cortisol concentrations were determined using a salivary cortisol enzyme immunoassay kit (Salivary Cortisol Elisa Kit, Salimetrics LLC, Pennsylvania, United States; Pagani et al., 2017; Marti et al., 2017), assessed in duplicate. The samples were thawed to room temperature, vortexed, and then centrifuged for 15 min at approximately 3,000 RPM (1,500 x g) immediately before performing the assay. Samples were tested for salivary cortisol using a high sensitivity enzyme immunoassay (Cat. No. 1-3002). Sample test volume was 25 μl of saliva per determination. The assay had a lower limit of sensitivity of 0.007 μg/dL, a standard curve range from 0.012-3.0 μg/dL, and an average intra-assay coefficient of variation of 4.6%, and an average inter-assay coefficient of variation 6.0%, which meets the manufacturers’ criteria for accuracy and repeatability in Salivary Bioscience,

Eye pictures were taken using an infrared thermography camera (FLIR E8-XT, FLIR Systems, Wilsonville, Oregon, United States) on d 33, 37, and 41 at approximately 0930-1000 h (following saliva sampling). Two researchers entered the calf’s pen to capture the pictures. One researcher held the calf gently but securely while the other took the pictures. Pictures were taken of both eyes (Bravo et al., 2018) at a 90° angle to the animal and approximately 0.5 m distance from the eye (Stewart et al., 2007). One to 5 images were taken/eye/calf/d, with the image numbers recorded for each calf/d. Each time a calf’s photos were taken, the ambient temperature and relative humidity were recorded from a Taylor Digital Indoor Thermometer Comfort Station (Canadian Tire, Guelph, Ontario, Canada) with the device positioned at the front of each calf’s pen. These values were later used during image analysis to account for atmospheric changes (Stewart et al., 2007; Bravo et al., 2018). The pictures were downloaded onto a computer and into the software program FLIR Tools (FLIR Systems, Wilsonville, Oregon, United States) for analysis. The clearest picture of each eye/calf/d was analyzed (Lecorps et al., 2018). The maximum temperature detected within an oval area traced around the eye, including the eyeball and approximately 1 cm surrounding the outside of the eyelids, was recorded (Figure 3.2; Stewart et al., 2007). The MET determined from both eye pictures was averaged to obtain 1 MET value for each calf/d.
Calves were blood sampled on d 34, 38, and 42 between 0830 and 1030 h. Ten mL of blood was collected from the jugular vein through a catheter (d 34) or venipuncture (d 38 and 42) using a 20 gauge 1 inch needle (greiner BIO-ONE, Kremsmünster, Austria) into a 10 mL vacutainer lavender top plastic blood collection tube with K2 EDTA (BD, Franklin Lakes, New Jersey, United States) which was gently inverted approximately 8 times. Immediately following, 4 mL of blood was poured into each of 2, 6 mL glass tubes (14-961-26, Fisher Scientific, Ontario, Canada) preloaded with 40 mg/tube of ascorbic acid (NOW Foods 100% Pure Ascorbic Acid Powder, Well.ca, Guelph, Ontario, Canada) and then mixed gently by inverting approximately 8 times. The ascorbic acid was added at 10 mg/mL, which preserves the blood by stabilizing and protecting the serotonin against oxidative loss and prevents platelet degradation, (Dr. Jimena Laporta, University of Wisconsin-Madison, Madison, Wisconsin, personal communication). The tubes were stored at -20°C until analysis (Marrero et al., 2019). The samples were shipped on dry ice to the University of Wisconsin-Madison where they were analyzed using a serotonin enzyme immunoassay kit (IM1749; Immunotec, Beckman Coulter, Marseille Cedex 9, France) according to the manufacturer’s instructions, assessed in duplicate (Marrero et al., 2019). The intra- and inter-assay CVs were 3.86% and 4.24%, respectively.

Lastly, samples of the MR and calf starter were collected at the beginning of each month and frozen at –28°C. Samples were later thawed overnight in the refrigerator and individually placed in a drying oven at 60°C for 48 h to determine the dry matter (DM) content. Samples of solid feed were ground through a 1-mm sieve (Model 4 Wiley Laboratory Mill, Thomas

3.2.5 Statistical Analyses

All statistical analyses were conducted using SAS 9.4 software (SAS Institute Inc., 2013). All data were imported into the statistical software program from Microsoft Excel (Microsoft Corp., Redmond, WA, USA). In SAS, data were assessed for normality using the UNIVARIATE procedure; all parameters were normally distributed. The GLIMMIX procedure of SAS was used for all analyses. Calf within batch and room was the experimental unit in all analyses and the
subject of the repeated statement for repeated measures models. Batch and room were considered as random effects. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

All data were collected over time, so all data were summarized by calf and day. The saliva cortisol, blood serotonin, cognitive test times, and lying data (number of bouts, average bout length, and lying time) data were obtained by calf and day. Average MET was determined by averaging the MET values determined for the left and right eyes. Total play duration was determined by summing the total time each calf spent running and rubbing shavings (per play assessment). The total play count was determined by summing the counts of bucks, kicks, jumps, head-shake/swings, and head-butts (per play assessment).

To address our objectives, the data for saliva cortisol, average MET, blood serotonin, total play duration and total play count were analysed in 3 steps. First, for each outcome, the baseline (pre-weaning) values for each treatment were compared using a mixed-effect linear regression model with the fixed effect of treatment. Next, for each outcome, the effect of treatment during the weaning period was tested using a repeated measures mixed-effect linear regression model, with the fixed effects of treatment, day, and the treatment by day interaction. Finally, for each outcome, to assess how those changed from baseline, the difference from baseline for each observation day was calculated and analyzed using a repeated measures mixed-effect linear regression model. The difference from baseline was calculated for each weaning value by subtracting each weaning day value from the corresponding baseline value.
Lying behavior (bouts, bout length, and time) data was analyzed using the same 3 steps outlined above except for the pre-weaning values, the effect of treatment was tested using a repeated measures mixed-effect linear regression model, with the fixed effects of treatment, day, and the treatment by day interaction. Lastly, the effect of treatment on the cognitive test results was tested using a repeated measures mixed-effect linear regression model, with the fixed effects of treatment, day, and the treatment by day interaction. For all of the repeated measures analyses, since all parameters had equal time spacing, the covariance strictures cs, csh, arh(1), ar(1), and un were tested and the one with the lowest BIC value was used for the analysis.

3.3 RESULTS

In the pre-weaning period, the number of lying bouts tended to vary by day (P = 0.09; Figure 3.3). As weaning progressed, the number of lying bouts/d decreased (P < 0.001). Compared to pre-weaning, there were fewer lying bouts during weaning (P < 0.001). In the pre-weaning period, the average lying bout length tended to vary by day (P = 0.07; Figure 3.3). As weaning progressed, the lying bout length increased (P < 0.001). Compared to pre-weaning, the lying bout length was greater during weaning (P < 0.001). In the pre-weaning period, total lying time varied by day (P = 0.006; Figure 3.3). In both the pre-weaning and weaning periods, CON calves tended to have a greater lying time than TLH calves (pre-weaning: CON = 1122.0 ± 10.85 min/d, TLH = 1096.1 ± 10.39 min/d, P = 0.09; weaning: CON = 1110.8 ± 8.56 min/d, TLH =
1088.4 ± 8.29 min/d, P = 0.06). Compared to pre-weaning, there was no change in lying time
detected during weaning (P ≥ 0.13).

No treatment difference was detected in the pre-weaning total play duration (P = 0.13;
Figure 3.4). During weaning, calves played for a longer duration (P < 0.001) on d 37 (11.4 ± 2.64
s) than on d 41 (0.2 ± 0.12 s). Compared to pre-weaning, on d 41, play duration was decreased (P < 0.001), while there was no change on d 37 (P = 0.30). No treatment difference was detected in
the pre-weaning total play count (P = 0.47; Figure 3.4). During weaning, there was a higher play
count on d 37 (15.7 ± 2.41) compared to d 41 (1.7 ± 0.50; P < 0.001). Compared to pre-weaning,
calves tended to have a higher play count on d 37 (P = 0.07), while they had a lower play count
on d 41 (P = 0.002). No treatment difference was detected in play duration and play count during
weaning (P ≥ 0.65).

There tended to be a treatment by day interaction (P = 0.07), whereby CON calves
completed the cognitive (detour) test faster on d 39 compared to d 38 (P = 0.04; Figure 3.5). No
change was detected in TLH calves between the 2 test days (P = 0.65) and no differences were
detected between treatments within day (P ≥ 0.15).

No treatment difference was detected in pre-weaning salivary cortisol (P = 0.93; Figure
3.6). During weaning (d 37 and 41) calves in the TLH treatment (0.16 ± 0.01 µg/dL) had higher
(P = 0.01) salivary cortisol than CON calves (0.11 ± 0.02 µg/dL). Compared to pre-weaning, on
both days during weaning salivary cortisol for the TLH calves was increased (P = 0.04), while
salivary cortisol for CON calves did not change ($P = 0.46$). There was no difference ($P = 0.75$) detected between the weaning days (d 37 and 41).

No treatment difference was detected in pre-weaning MET ($P = 0.18$; Figure 3.7). Calves on the TLH treatment (37.2 ± 0.26 °C) tended ($P = 0.08$) to have a higher MET than CON calves (36.7 ± 0.26 °C) during weaning. There was no difference ($P = 0.69$) detected between the weaning days (d 37 and 41). No changes from pre-weaning to weaning in MET were detected ($P > 0.15$).

No treatment difference was detected in pre-weaning blood serotonin ($P = 0.27$; Figure 3.8). No treatment or day effects during weaning in blood serotonin were detected ($P > 0.28$). No changes from pre-weaning to weaning in blood serotonin were detected ($P > 0.60$).

### 3.4 DISCUSSION

It is important to note that time is confounded in the weaning results interpretations, although due to weaning being an event with many implications on the animal including psychological and physical, as described in the introduction, we feel it is safe to assume it is weaning, and not age, that is attributable to the results. As hypothesised, weaning resulted in behavioral changes, including lying and play behavior, compared to pre-weaning. Compared to pre-weaning, calves had fewer lying bouts and the length of those lying bouts was greater during weaning. As weaning progressed, the number of lying bouts/d further decreased and the lying bout length increased. Fewer, but longer, lying bouts may reflect a change in eating behavior.
Specifically, as the calves progressed in weaning, due to reduced milk intake, the calves would have needed to consume more solid feed (Khan et al., 2007), which likely influenced their eating behavior (not measured) and, consequently, lying behavior. Despite these lying bout changes, daily lying time was not affected by weaning. Based on other weaning studies it was expected that the calves would spend more time standing and, therefore, less time lying during weaning because weaning stress is associated with more standing (and less lying) behavior in calves (Budzynska and Weary, 2008; Overvest et al., 2018; Yeste et al., 2020). During weaning, the combination of receiving less milk and not consuming enough solid feed (< 800 g/d) resulted in the calves not meeting their energy and protein requirements (Olmeda et al., in preparation). It is possible that any increased standing behavior associated with stress was balanced off with increased lying behavior associated with insufficient energy and nutrient intake, resulting in no detected change in total lying time due to weaning.

Play behavior has been reported to be an indicator of positive affective state in species that are normally playful (Ahloy-Dallaire et al., 2018), as animals are motivated to play when their basic needs are met (Held & Špinka, 2011). Stressors, including weaning, have been reported to result in reduced play behavior in dairy calves (Krachun et al., 2010; Miguel-Pacheco et al., 2015). As weaning progressed, calves played for a shorter duration and had a lower play count on d 41 compared to d 37. On d 37, the calves had just begun the second phase of weaning (3 L/d of milk), while on d 41, the calves were on their third day of the last weaning phase (0.4 L/d of milk). Likely reasons for the reduced play activity on d 41 include the calves not meeting
their protein and energy requirements, which would be conducive for physical activity, as well as presumed increased psychological stress from being further along in the weaning process (Jasper et al., 2008; Krachun et al., 2010). Interestingly, compared to pre-weaning, calves tended to have a higher play count on d 37, while they had a lower play count on d 41. The increase in play count behaviors on d 37 may be because some behaviors, such as kicking and head-shaking/swinging, were actually performed due to frustration associated with weaning rather than playfulness (Kiley-Worthington, 1983).

To our surprise, there were few detected changes in the physiological parameters due to weaning. Saliva cortisol has been reported to increase in calves following various stressors; mean salivary cortisol levels were reported as 0.07 µg/dL in calves separated from their foster cow without any step-down weaning method (Loberg et al., 2008): 0.14 µg/dL after 20 h of transport without a rest stop (Marti et al., 2017), and 3.16 µg/dL following a dystocic birth (Kovács et al., 2021). In the present study, the mean salivary cortisol concentration in the pre-weaning period was 0.12 ± 0.01 µg/dL, which falls within the range of salivary cortisol literature values reported for presumably stressed calves. This would potentially indicate hypothalamic-pituitary-adrenal axis activation throughout the observation period, from different stress or other factors (Mormède et al., 2007).

When the sympathetic nervous system is activated, blood flow to the eye may be increased, and thus, higher MET may be detected, especially in the lacrimal caruncle region (Stewart et al., 2007; Dai et al., 2015). No studies were identified that assessed MET in response
to weaning stress, although MET has been reported to increase in calves following various stressors including castration (Stewart et al., 2010), following loading into a trailer (Lecorps et al., 2018), and following a 3 h transport (Bravo et al., 2018). Although the affective state of the calves appeared to be more negative during weaning based on the behavioral results, weaning did not elicit increased eye temperature, perhaps because the stress was not great enough to elicit an effect (Dai et al., 2015). It is possible that different weaning methods, such as weaning off a cow, or abrupt weaning (no step-down), could cause a greater stress stimulus that results in increased MET, although more investigation is needed.

Lower serotonin concentration in whole blood or serum has been associated with more negative affective states in humans (Cleare et al., 1997), dogs (Rosado et al., 2010), chickens (de Haas et al., 2013), and calves (Marrero et al., 2021). Therefore, we predicted the dairy calves would have lower blood serotonin concentration during weaning compared to pre-weaning. Despite behavioral signs of a more negative affective state during weaning, there was no detected change in blood serotonin concentration. Likewise, Riggio et al. (2021) reported serum serotonin levels were not associated with the behavioral response of dogs to a stressful situation. Yeste et al. (2020) reported no effect of serotonin precursor (tryptophan) supplementation on dairy calf behavior or stress markers (serum cortisol and haptoglobin) at weaning, although they did not report actual blood serotonin levels. Perhaps there was no detected change in blood serotonin at weaning because different stressors cause different biological responses (Moberg and Mench, 2000) and the stress elicited by the weaning conditions in the present study did not
impact blood serotonin concentration during the week of weaning. Notably, the physiological functions of serotonin are still not fully understood (Bacqué-Cazenave et al., 2020). Serotonin has not been well researched to date in dairy calves, and to our knowledge, there are no other calf studies in the literature that assess the effect of weaning on blood serotonin levels. Clearly, more investigation is required to explore the effect of weaning, and associated impacts on calf affective state, on dairy calf blood serotonin levels.

Some probiotic study results are included in the discussion of the TLH results because there is less literature in this area on postbiotics and they may have similar effects, as explained in the introduction. Calves on the TLH treatment tended to have less lying time throughout the entire study. While more standing may be associated with stress, it is also possible that the standing time was associated with more activity. In support of this, Kelsey et al. (2018) reported that beef cows supplemented with a probiotic had higher activity. Furthermore, increased standing in our study calves could also be associated with greater eating time, possibly as a result of increased exploration of feed or greater feed intake. Although there were no detected treatment effects by Olmeda et al. (in preparation) on feed intake during our study periods, they did report that TLH calves had higher feed intake during the post-weaning period (specifically from d 43 - 56).

It was predicted that the calves would complete the cognitive test (detour task) faster on d 39 compared to d 38 (i.e., demonstrating learning). While this was observed in the CON calves, there was no change observed in TLH calves. Although notably, no differences were detected
between the treatments in terms of how fast they completed the detour task on either of the test days. Stress can reduce cognitive ability (Liang et al., 2015), due to stress hormones, in particular, cortisol (Joels et al., 2018). Interestingly, during weaning, calves supplemented with TLH had higher salivary cortisol levels than CON calves, which increased from pre-weaning in those calves fed the TLH. Additionally, TLH calves tended to have a higher MET than CON calves during those days of weaning. These results suggest that the TLH supplementation may have resulted in those calves having a greater reaction to the weaning stress. Part of that reactivity, as noted in the increase in salivary cortisol and MET, may have also been related to the increased standing (and possibly overall activity levels; Stewart et al., 2010; Ede et al., 2019) of those TLH calves.

There were no treatment effects detected on blood serotonin, although numerically, blood serotonin concentration was greater in TLH calves throughout the trial. Stress may cause elevated serotonin for serotonergic homeostasis (Chaouloff et al., 1999; Andrews et al., 2015), which would further support a more negative affective state in the TLH calves. Alternately, it is possible that the TLH product increased serotonin production. As reported in the introduction, although a different species was used, Hara et al. (2018) reported mice supplemented with heat-killed Lactobacillus casei subsp. casei 327, had higher serotonin levels in colon tissue compared to control mice. Although not reported, those mice may have had higher blood serotonin also, as the majority of the serotonin in the body is produced in the gut (Jenkins et al., 2016), and then released into the blood (Mawe and Hoffman, 2013)
Overall, the detected effects of TLH supplementation were not completely expected, as it seems TLH supplementation may be associated with indicators of a more negative affective state in those calves at weaning. Olmeda et al. (in preparation) reported there were no treatment effects on gut permeability, or microbial diversity, supporting that the TLH effects were independent of those factors, and that tyndallized probiotics may have effects on the gut-brain axis through non-viable probiotic cells and metabolites, without affecting the gut microbiota. This is further supported by the results and proposed mechanisms of Maehata et al. (2019), outlined in the introduction of the present paper. Maehata et al. (2019) was the only study identified in the literature that supplemented and investigated the effects of TLH on mental well-being in animals.

These results with postbiotic supplementation to dairy calves do not correspond with the results on probiotic supplementation previously reported in the literature. Zhang et al. (2016) reported that during weaning there was no effect of probiotic (Lactobacillus plantarum and Bacillus subtilis) supplementation on adrenaline and creatine kinase activity, although, it was associated with an increased T-lymphocyte transformation rate and decreased blood serum cortisol compared to calves not receiving any probiotics. Similarly, Xie et al. (2020) reported that supplementation of a herbal tea residue fermented by lactic acid bacteria was associated with reduced serum cortisol in heat stressed calves. Likewise, Lee et al. (2019) reported that supplementation of Saccharomyces boulardii was associated with a lower heart rate during a thermal neutral period, and lower cortisol during a heat stress period in dairy calves.
Alternatively, Bayatkouhsar et al. (2013) reported no effect of multi-strain probiotic blends on blood plasma cortisol following weaning. Kelsey et al. (2018) also investigated behavioral outcomes and reported supplementation of a probiotic blend (*Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus plantarum*) was associated with a slower chute exit speed in beef cows and their nursing calves. Likewise, Kelsey et al. (2018) also reported that during a novel object test, probiotic supplementation was associated with a slower arena exit speed in weaned calves, and higher activity and less vocalizations in cows. Although, Kelsey et al. (2018) reported no detected effect of the probiotic on serum cortisol concentrations in the cows. It is possible that probiotics, as were used in these studies, exert more beneficial effects through the gut-brain axis than postbiotics in dairy calves. As this is the first study, to our knowledge, to investigate the effect of postbiotics on dairy calves’ affective state, and as only one postbiotic was investigated, it is difficult to draw such a conclusion. As well, this was the first study, to our knowledge, that investigated the effects of the species LH on dairy calves’ indicators of stress. Thus, more research is needed to confirm LH’s, and in overall -postbiotic effects.

There are some notable limitations to the current study. Firstly, weaning and calf age were confounded; there was no way for us to distinguish the results between them. To solve this, in future studies, researchers could have an additional treatment of calves that is not weaned to identify if their behavior changes as it did to the calves during weaning in the present study. Only one dose of LH /d was tested; it is possible that higher dosages would result in different
outcomes (Zagórska et al., 2020). The last phase of weaning (0.4 L/d) was included to allow continued administration of the postbiotic using the same method (via milk), although administration of such small amounts of milk is not a common practice in industry and could have affected the physiological and behavioral outcomes observed. Therefore, perhaps stopping milk feeding and LH supplementation following the 3 L/d weaning phase would provide results that are more applicable to industry. Furthermore, extreme measures were taken to minimize transfer of microbiota between calves, including changing gloves and scrubbing boot covers in disinfectant when moving between calves. This is not realistic nor common in industry and, therefore, may have resulted in different microbial communities than what may be observed in commercial settings, which could affect the results.

3.5 CONCLUSION

Weaning dairy calves resulted in behavioral changes indicative of a negative affective state, including fewer but longer lying bouts and reduced play behavior, with no detected changes in physiological measures. Supplementation of TLH to calves was associated with reduced lying time throughout the study, and increased salivary cortisol and a tendency for increased MET during weaning. More research on LH supplementation, either tyndallized or in live form, to dairy calves is required to confirm and understand its effects.
3.6 ACKNOWLEDGMENTS

Thank you to all the staff at the Ponsonby General Animal Facility (Ponsonby, Ontario, Canada) and Ontario Dairy Research Centre (Elora, Ontario, Canada) for their part in caring for the calves. Thank you to Brooke Boonstoppel, Claudia Jaczkowski, Katherine Perry, and Sabina Czachor of the University of Guelph (Guelph, ON, Canada) for their assistance with sample collection and analysis. Thank you also to Veronica Fursova, Aly Hoste, Claudia Campoli, Junyu Zhang, Kehan Zhang, and Gary Cotte of the University of Guelph (Guelph, ON, Canada) for their assistance feeding and care for the calves. Thank you also to Dr. Jaeju Yu of the University of Guelph (Guelph, ON, Canada) for laboratory consultation, and to Dr. Laura Hernandez and Waneska Spinelli Frizzarini of the University of Wisconsin-Madison (Madison, Wisconsin, United States) for conducting the blood serotonin assays. Thank you to Lallemand (Montreal, QC, Canada) and particularly Dr. Clothilde Villot (Lallemand) for project support. This project received support from the Natural Sciences and Engineering Research Council of Canada (Ottawa, ON, Canada) and the Ontario Agri-Food Innovation Alliance Research Program of the University of Guelph and the Ontario Ministry of Agriculture, Food, and Rural Affairs (Guelph, ON, Canada). The authors have not stated any conflicts of interest.
Table 3.1. Ingredient and chemical composition (mean ± SD) of the calf starter and milk replacer fed to all calves.

<table>
<thead>
<tr>
<th>Ingredient, % in ration</th>
<th>Calf starter feed¹</th>
<th>Milk replacer²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf starter pellet³</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>Steam flaked corn</td>
<td>36</td>
<td>-</td>
</tr>
<tr>
<td>Molasses</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td><strong>Chemical Composition⁴</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM, % DM</td>
<td>88.5 ± 0.55</td>
<td>97.0 ± 0.74</td>
</tr>
<tr>
<td>CP, % DM</td>
<td>20.4 ± 1.08</td>
<td>27.0 ± 0.66</td>
</tr>
<tr>
<td>NDF, % DM</td>
<td>10.7 ± 0.61</td>
<td>-</td>
</tr>
<tr>
<td>ADF, % DM</td>
<td>5.2 ± 0.91</td>
<td>-</td>
</tr>
<tr>
<td>Crude Fibre, % DM</td>
<td>-</td>
<td>0.6 ± 0.29</td>
</tr>
<tr>
<td>Ash, % DM</td>
<td>7.2 ± 0.36</td>
<td>7.0 ± 0.22</td>
</tr>
<tr>
<td>NFC, % DM</td>
<td>57.3 ± 0.67</td>
<td>-</td>
</tr>
<tr>
<td>Starch, % DM</td>
<td>41.3 ± 0.94</td>
<td>-</td>
</tr>
<tr>
<td>Fat⁵, % DM</td>
<td>2.2 ± 0.27</td>
<td>19.0 ± 1.44</td>
</tr>
<tr>
<td>Lactose⁶, % DM</td>
<td>-</td>
<td>47.1 ± 1.44</td>
</tr>
<tr>
<td>Ca, % DM</td>
<td>1.4 ± 0.14</td>
<td>1.0 ± 0.05</td>
</tr>
<tr>
<td>P, % DM</td>
<td>0.4 ± 0.04</td>
<td>0.7 ± 0.02</td>
</tr>
<tr>
<td>Na, % DM</td>
<td>0.4 ± 0.02</td>
<td>0.6 ± 0.06</td>
</tr>
</tbody>
</table>

¹Texturized calf starter feed was supplied by Shur-Gain (Ontario, Canada).
²Milk replacer was supplied by Grober Nutrition (Cambridge, Ontario, Canada).
³Calf starter pellet included ingredients: Soy meal, soft-ground wheat, barley, gluten, calcium carbonate, calf micro premix, salt, magnesium oxide, and dicalcium phosphate.
⁴Chemical analysis was done by A&L Laboratory Services Inc (London, ON, Canada).
⁵Fat for the rations is crude fat. Fat for the milk replacer was done by an acid hydrolysis test (AOAC, 1995: Method 954.02).
⁶Lactose is assumed to be 100-CP-Fat-Ash.
Table 3.2. Description of recorded play behavior. adapted from that of previous studies (Jensen et al., 1998; Mintline et al., 2013).

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locomotor</td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Includes:</td>
</tr>
<tr>
<td></td>
<td>- Trot: Two-beat gait, with leg movements synchronized diagonally</td>
</tr>
<tr>
<td></td>
<td>- Canter: Three-beat gait in between a trot and a gallop</td>
</tr>
<tr>
<td></td>
<td>- Gallop: Four-beat gait with a phase where all legs are off the ground</td>
</tr>
<tr>
<td>Buck</td>
<td>Both rear legs are lifted off the ground and kicked in the rear direction</td>
</tr>
<tr>
<td>Kick</td>
<td>One rear leg is lifted off the ground, sometimes tucked in under the body first, and then extended to the rear or side</td>
</tr>
<tr>
<td>Jump</td>
<td>The two forelegs are lifted from the ground, as the forepart of the body is elevated, during the last phase of the movement, the hind legs may be lifted from the ground</td>
</tr>
<tr>
<td>Turn</td>
<td>The two forelegs are lifted from the ground, as the forepart of the body is elevated and turned to one side, followed by the calf’s rear-end being elevated and moving sideways to fall into alignment with the front end</td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
<tr>
<td>Head-shake/swing</td>
<td>The head is shaken, swung or rotated</td>
</tr>
<tr>
<td>Rub shavings</td>
<td>Rubbing head, throat or neck in shavings in a playful manner, kneeling down on the two forelegs</td>
</tr>
</tbody>
</table>
**Figure 3.1.** Cognitive test (detour task) pen layout. A = pen entrance, B = detour apparatus.
**Figure 3.2.** Example of a calf eye infrared thermography image used to determine maximum eye temperature. The triangle placement is where the software program (FLIR Tools) detected is the maximum temperature within the circle placed around the calf’s eye (lacrimal caruncle).
Figure 3.3. The number of lying bouts (count/d; a), the average lying bout length (average min/bout; b), and the time spent lying (min/d; c) by treatment and day (mean ± SE). The treatments include: CON = control (calves received no *Lactobacillus helveticus*; n = 11) and TLH = calves received 5 g/d of tyndallized *Lactobacillus helveticus* at $10^9$ CFU/g, split over 2 milk feedings from d 3-42 (n = 12). The vertical dashed line indicates when weaning began (d 35).
Figure 3.4. Total play duration (s/d; a), and total play count (count/d; b) by treatment and d 
(mean ± SE). The total play duration was determined by the total time the calf spent running 
and/or rubbing shavings, while the total play count was determined by the total count of bucks, 
kicks, jumps, head-shake/swings, and head-butts the calf performed (all behaviors defined in 
Table 2) during a 210 second play assessment conducted around the time of bedding (0900 h). 
The treatments include: CON = control (calves received no Lactobacillus helveticus; n = 11) and 
TLH = calves received 5 g/d of tyndallized Lactobacillus helveticus at 10⁹ CFU/g, split over 2 
milk feedings from d 3-42 (n = 12). The vertical dashed line indicates when weaning began (d 
35).
Figure 3.5. Cognitive test duration (mean ± SE) by treatment and d. The treatments include: CON = control (calves received no Lactobacillus helveticus; n = 11) and TLH = calves received 5 g/d of tyndallized Lactobacillus helveticus at $10^9$ CFU/g, split over 2 milk feedings from d 3-42 (n = 12).
Figure 3.6. Saliva cortisol (mean ± SE) by treatment and d. The treatments include: CON = control (calves received no *Lactobacillus helveticus*; n = 11) and TLH = calves received 5 g/d of lyophilized *Lactobacillus helveticus* at $10^9$ CFU/g, split over 2 milk feedings from d 3-42 (n = 12). The vertical dashed line indicates when weaning began (d 35).
Figure 3.7. Maximum eye temperature (mean ± SE) by treatment and d. The treatments include: CON = control (calves received no *Lactobacillus helveticus*; n = 11) and TLH = calves received 5 g/d of tyndallized *Lactobacillus helveticus* at 10⁹ CFU/g, split over 2 milk feedings from d 3-42 (n = 12). The vertical dashed line indicates when weaning began (d 35).
Figure 3.8. Blood serotonin (mean ± SE) by treatment and d. The treatments include: CON = control (calves received no *Lactobacillus helveticus*; n = 11) and TLH = calves received 5 g/d of tyndallized *Lactobacillus helveticus* at $10^9$ CFU/g, split over 2 milk feedings from d 3-42 (n = 12). The vertical dashed line indicates when weaning began (d 35).
4 CHAPTER 4: GENERAL DISCUSSION

4.1 Important Findings

The overall aim of this thesis was to investigate the effects of dietary supplements on dairy calf health and welfare. I addressed this with 2 studies, one focused on supplementing *Echinacea purpurea* (EP; Chapter 2) and the other focused on supplementing tyndallized *Lactobacillus helveticus* (TLH; Chapter 3).

The first study (Chapter 2) was focused on immunity, health, intake, and growth outcomes of supplementing EP to dairy calves kept at a rearing facility. This was investigated through 2 treatments: EP supplementation from d 14-28 (E14) and 1-56 (E56) of life. The E14 treatment was chosen because we were interested to see if a targeted supplementation of EP from d 14 to 28 following arrival to the rearing facility would be as beneficial as supplementing it across the whole milk-fed period (E56). Day 14-28 was chosen based on the estimated age of the calves at arrival to the facility (5-14 d of age) and when respiratory illness is most commonly detected in pre-weaned dairy calves (Windeyer et al., 2014; Urie et al., 2018).

Supplementation of EP was associated with immunomodulation and reduced inflammation, evidenced through lower haptoglobin levels, segmented neutrophil counts, segmented neutrophil/lymphocyte ratio and higher lymphocyte counts compared to calves not receiving EP. Despite those changes, only few health and growth improvements were associated with EP supplementation, including lower respiratory scores in auction-derived calves and
greater post-wean weekly BW in calves with a heavier arrival BW, compared to calves not receiving EP. These outcomes were more prominent in calves fed EP throughout the whole milk feeding period. These results of Chapter 2 were overall expected, as most detected effects were indicative of the EP being beneficial to the dairy calves, which aligns with the majority of the results of previous EP supplementation studies to livestock species.

Meanwhile, the second study (Chapter 3) was focused on the effect of weaning on dairy calf affective state and if supplementing TLH would reduce those indicators of negative affect during weaning. Weaning resulted in fewer, but longer lying bouts and reduced play in calves, indicative of a more negative affective state compared to pre-weaning. Supplementation of TLH resulted in mixed findings, although measures indicative of a more negative affective state, including less lying time throughout the study and higher salivary cortisol and MET during weaning, were observed in supplemented calves. The behavioral indicators of a more negative affective state in the calves at weaning were expected due to weaning stress (Jasper et al., 2008). Alternatively, the TLH supplemented calves had some physiological indicators of a more negative affective state than the control calves was unexpected. This is because the literature generally supports that microbial-based dietary supplements (probiotics and postbiotics), including Lactobacillus helveticus, are generally beneficial to the mental well-being of rodents and humans (Messaoudi et al., 2011; Maehata et al., 2019).

Taken together, these results support that the effects of a supplement on the health and welfare of dairy calves depends on the specific supplement used, including how it was prepared,
the genre, species, and strain, the supplementation methods (i.e., timing/duration), and the history and condition of the supplemented animals (i.e., source, health, BW, etc.). The manufacturing of a supplement can affect its bioactive components and consequent effects on the animal consuming it. Considering EP, researchers have reported growing methods (i.e., growing medium, and plant age at harvest), the plant part used, and the extraction method affect the active component profile of the EP (Dehkordi et al., 2011). Likewise, considering postbiotics, different methods of inactivation of live probiotics can affect their structural components (Taverniti and Guglielmetti, 2011). The specific genre, species, and strain of the supplement are also important to consider, as they can have different properties (Barnes et al., 2005; Cuevas-González et al., 2020). The active component profile of the EP supplemented in Chapter 2 differed from that used in other calf studies, including that described by Ayrle et al. (2021). The strain of TLH used in Chapter 3 was different from that used in comparable studies, including that described by Maehata et al. (2019). Consequently, I detected some different results from those previous studies. Considering supplementation methods, only one TLH treatment was investigated in Chapter 3, that being for the whole milk-fed period (d 3-42), preventing comparison with other supplementation regimes. In Chapter 2, two EP supplement durations were investigated. Some results differed between the two durations, supporting that the duration of supplementation matters. For example, calves in the E56 treatment tended to have lower levels of segmented neutrophils, and of calves with a heavier arrival BW, E56 calves had higher average post-wean weekly BW, compared to control calves (not receiving EP), while no such differences were detected for calves in the E14 treatment. These results support a longer duration of
supplementation of EP can equate to more effects on the calves. Finally, taking into account the history and condition of the study animals also had impacts on the results in both Chapter 2 and 3. For example, in Chapter 2, in the milk-fed period, respiratory health was dependent on the source of the calves, whereby respiratory health was improved in auction-sourced calves supplemented EP although conversely, respiratory health was worse in drover sourced calves supplemented EP. While we did not detect any health-related differences in the calves between the 2 sources, there were likely underlying factors involved, possibly genetics or care prior to arrival to the study facility. The calves were sourced from the same farm in Chapter 3 and all managed the same, eliminating that factor. It is possible the results in Chapter 3 could have been different if calves were sourced from more than one farm, as those early life farm specific factors, such as neonatal care practices and microbial communities, can impact calf health and welfare short and long term (Hulbert and Moisa, 2016). For example, if calves from one source experienced more stress, it is possible the effects of a supplement may be more noticeable (Uyeno et al., 2015). These considerations help explain the variation seen between the results of these studies and comparable studies.

4.2 Limitations and Future Research

While several interesting results were generated in Chapter 2 and 3, caution must be exercised when applying those results due to limitations of both studies. Additionally, the results of this thesis provide insight on areas that need additional work in the future. One identified limitation, common to both of the studies, is that only male, Holstein calves were enrolled. It is
possible that the outcomes of a supplement could be different between both sex and breeds due to physiological and physical differences. Therefore, future work should investigate supplementation of these products in dairy calves in both sexes and different dairy breeds (e.g. Jersey, Ayrshire, Brown Swiss), and include those factors as covariates in the analysis. Due to time and resource limitations, as well as the fact that male calves were used who were destined for other calf growing facilities to prepare them for market soon after weaning, I was unable to follow the calves long term (i.e., up to 1 year of age or longer), to investigate if there were long-term effects of the supplements on the calves. In future studies, especially those conducted using female dairy calves who are more likely to remain on farm (rather than leave for the veal or dairy-beef industry), the calves should be followed up to and through the first lactation, to investigate if there are long-term effects, including on health, milk production, and reproduction. Also due to time and resource limitations, we were unable to investigate many dosing regimes (daily dosage, timing, and duration of supplementation). The amount and duration of administration of a supplement will affect the results due to varying amounts of the supplement and, therefore, also its active components present in the animal’s body (Zagórska et al., 2020). For supplements that successfully elicit beneficial effects, it is important to try to identify the regime that will maximize those benefits and make it a worthwhile investment. Therefore, in future studies, more treatment regimes should be investigated considering daily dose, timing, and duration of supplementation. Finally, detailed preparation methods of the supplements were unknown, as discussed above. This can impact the active component profile of the supplement. Therefore, researchers conducting studies on supplements in the future should make sure they
can obtain and report supplement preparation methods and active component profiles as much as possible, to advance what we know about this link, and through study comparisons, identify the preparation conditions that create the most efficient supplement to maximize beneficial effects on the animal.

Other limitations were study specific. In Chapter 2, the exact ages and health history and care of the calves prior to arrival to the facility for enrollment in the study were unknown, factors known to affect calf immunity and health. While I did my best to control for differences, including their serum total protein, arrival BW, and their source, future work should ensure exact calf ages are known, and account for them in the analysis, or ensure that calves are enrolled in the study at the same age. Due to facility design, the calves were unable to be fed solid feed individually (but rather fed in groups of 5 calves), preventing individual calf feed intakes from being obtained. This meant I had to report feed intake and feed conversion rate on a group basis, which is not as accurate because each calf varies in the feed intake and growth. Therefore, in future studies, the facility should be set up to allow individual calf feed intakes to be obtained.

In Chapter 3, the final phase of weaning (0.4 L/d), included to allow continued administration of the postbiotic, is not common in industry, perhaps reducing the applicability of the results to industry. The continued administration might have changed the results, as it is possible that the small amounts of milk fed (0.4 L/d) may have served as a tease to the calves that resulted in more stress than if they were not offered any milk following the 3 L/d weaning phase. To explain, the taste of milk elicits sucking behavior in dairy calves (Rushen and de
Passille, 1995). Offering such a small amount of milk may have had that effect, followed by quick removal of the buckets, possibly resulting in increased stress from the motivation to suck without a proper outlet and frustration from not receiving a complete milk meal; as we were measuring affective state, this would be pertinent to the results. Therefore, for future studies, complete weaning and TLH supplementation after the 3 L/d weaning phase should be done to eliminate the possible increased stress from a milk ‘tease’, better match industry practices, and therefore make the results more applicable to commercial settings. Additionally, extreme hygiene measures were taken to minimize microbial transfer between calves - this was done because a fellow researcher was investigating the effect of the TLH on the calves’ gut microbiota composition - although the extreme hygiene may have resulted in different microbial communities than what may be observed on commercial farms. In the future, depending on the study objectives, researchers should consider aligning the hygiene precautions used with that typical on commercial farms to ensure better applicability. Finally, brain serotonin may be a better indicator of affective state than blood serotonin. It is well known that there is an association between brain serotonin and affective state (Marrero et al., 2019), while less research is available on the association between blood serotonin and affective state, although there is research that supports an association in humans and animals (Cleare et al., 1997; de Haas et al., 2013). The distinction between brain and blood serotonin is because serotonin cannot cross the blood brain barrier (Namkung et al., 2015). In the Chapter 3 study, brain samples were collected from a subset of calves during dissections that occurred at the end of weaning (d 43) to later be analyzed for serotonin concentrations. Due to time constraints, the brain sample results have not
been received yet, and therefore could not be included in this thesis, although they will be analyzed and included later on. Therefore, researchers investigating the effect of a supplement on affective state in dairy calves should consider obtaining brain serotonin concentrations also.

Another idea for future research is measuring water intake. Water intake would be an interesting measure to consider because it is associated with higher feed intake (Parsons et al., 2022). There is limited literature available on the effects of EP on dairy calves including immunity, health, intake, and growth measures. To our knowledge, there have been no studies conducted to date investigating the effects of TLH supplementation on measures indicative of affective state in dairy calves. Therefore, more research is needed in these areas to clarify the biological modes of action and effects on dairy calves.

4.3 Implications

Dietary supplements are a promising area in dairy calf rearing, which hold great potential to improve dairy calf health and welfare, while reducing the use of antimicrobials. These outcomes are important to make farms more profitable and support public perception and industry sustainability. The results of my thesis research support that EP supplementation can reduce inflammation and stimulate immunity in dairy calves, and therefore would be recommended as a supplement for dairy calves on commercial farms to support immunity and health. My research also demonstrated dairy calves experience a more negative affective state at weaning, especially as weaning progresses, and therefore efforts should be taken to minimize
weaning stress to support their welfare. Finally, the effects of TLH supplementation on dairy calf affective state are inconclusive, and thus its supplementation would not be recommended at this time. This research contributes to filling some gaps in dairy calf health and welfare research and serve as valuable comparisons for future research.
REFERENCES


Ayrle, H., M. Mevissen, M. Kaske, H. Nathues, N. Gruetzner, M. Melzig and M. Walkenhorst. 2016. Medicinal plants – prophylactic and therapeutic options for gastrointestinal and


https://doi.org/10.1111/jvim.12531.


Granados-Chinchilla, F. 2017. A review on phytochemicals (including essential oils and extracts) inclusion in feed and their effects on food producing animals. J. Dairy Vet. Sci. 3. DOI: 10.19080/JDVS.2017.03.555620.


Haron, M. H., H. L. Tyler, N. D. Pugh, R. M. Moraes, V. L. Maddox, C. R. Jackson, and D. S. Pasco. 2016. Activities and prevalence of proteobacteria members colonizing *Echinacea*


Liang, S., T. Wang, X. Hu, J. Luo, W. Li, X. Wu, Y. Duan, and F. Jin. 2015. Administration of *Lactobacillus helveticus* NS8 improves behavioral, cognitive, and biochemical aberrations


Ohland, C. L., L. Kish, H. Bell, A. Thiesen, N. Hotte, E. Pankiv, and K. L. Madsen. 2013. Effects of Lactobacillus helveticus on murine behavior are dependent on diet and genotype
http://dx.doi.org/10.1016/j.psyneuen.2013.02.008.


http://dx.doi.org/10.3390/ani10010116.


https://doi.org/10.3168/jds.2020-19689.


Steinmuller, C., J. Roesler, E. Grottrup, G. Franke, H. Wagner, and M. Lohmann-Matthes. 1993. Polysaccharides isolated from plant cell cultures of Echinacea purpurea enhance the resistance of immunosuppressed mice against systemic infections with Candida albicans and


of essential oil in Echinacea purpurea L. Pak. J. Pharm. Sci. 26:403-408.
probiotics to psychobiotics – the gut-brain axis in psychiatric disorders. Benef. Microbes. 11:
Zamojska, D., A. Nowak, I. Nowak, and E. Macierzynska-Piotrowska. 2021. Probiotics and
postbiotics as substitutes of antibiotics in farm animals: a review. Anim. 11:3431.
https://doi.org/10.3390/ani11123431.
Zhang, R., M. Zhou, Y. Tu, N. F. Zhang, K. D. Deng, T. Ma, and Q. Y. Diao. 2016. Effect of
oral administration of probiotics on growth performance, apparent nutrient digestibility and
https://doi.org/10.1111/jpn.12338.
2020. A dynamic mouse peptidome landscape reveals probiotic modulation of the gut-brain
https://doi.org/10.1038/s41423-020-0402-2.