The Effects of Processing Diets for Companion Animals

by

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ABSTRACT

THE EFFECTS OF PROCESSING ON DIETS FOR COMPANION ANIMALS

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For my thesis, I investigated the effects of various treatments on companion animal diets. I first assessed how soaking and steaming affected nutrient contents in hay, and how feed preferences and glycemic responses of Standardbred racehorses were influenced by these processes. Soaking for 30min reduced soluble carbohydrates and potassium, while steaming for 60min conserved these nutrients. Horses’ glycemic responses were not affected by processing, and they preferred dry and steamed hay to soaked hay; therefore, we concluded that steaming is an effective treatment to conserve nutrients in hay for performance horses. In my second study, I sought to determine whether methionine hydroxy analogue (Alimet) mitigates pathogens in raw ground meat. Including Alimet as 1.25% reduced Salmonella enterica and Listeria monocytogenes by 20% and 26%, respectively. Overall, the work in this thesis has demonstrated how simple changes can improve the nutritional integrity, hygienic quality, and palatability of existing companion animal diets.
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<th>Description</th>
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<tr>
<td>ADF</td>
<td>Acid detergent fibre</td>
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<tr>
<td>and</td>
<td>α-neutral detergent fibre</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BW</td>
<td>Body weight</td>
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<td>CFU</td>
<td>Colony forming unit</td>
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<tr>
<td>CP</td>
<td>Crude protein</td>
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<tr>
<td>DLM</td>
<td>DL-methionine</td>
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<tr>
<td>DM</td>
<td>Dry matter</td>
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<tr>
<td>DMB</td>
<td>Dry matter basis</td>
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<tr>
<td>EMS</td>
<td>Equine metabolic syndrome</td>
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<tr>
<td>ESC</td>
<td>Ethanol-soluble carbohydrates</td>
</tr>
<tr>
<td>HYPP</td>
<td>Hyperkalemic periodic paralysis</td>
</tr>
<tr>
<td>MHA</td>
<td>Methionine hydroxy analogue</td>
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<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<tr>
<td>NSC</td>
<td>Non-structural carbohydrates</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PPID</td>
<td>Pituitary pars intermedia dysfunction</td>
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<tr>
<td>PSSM</td>
<td>Polysaccharide storage myopathy</td>
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<tr>
<td>RMBD</td>
<td>Raw meat-based diet</td>
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<tr>
<td>SP</td>
<td>Soluble protein</td>
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<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
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<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
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<td>WSC</td>
<td>Water-soluble carbohydrates</td>
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1. General Introduction

One of the major areas of study within the integrated science of animal nutrition is the concept of optimizing the feeds that we provide to our animals. This improvement of existing fodder is done by investigating the effects of various treatments on these feeds. A diet or foodstuff may be modified to improve attributes such as: digestibility, palatability, physical characteristics, the balance of nutrient profile of the feed to better suit the needs of the animal consuming it, or a reduction of pathogenic and spoilage bacteria. These modifications of animal diets can be found across the animal nutrition world, including agricultural, companion, and exotic animal nutrition (Mathison, 1996; McGoogan & Gatlin, 1997; Teaford & Oyen, 1989; Tran et al., 2008).

One common example of processing is the application of heat, which is used to improve the digestibility of plant-based ingredients (Carré et al., 1991; Conan & Carre, 1989). Plant-based ingredients, such as legumes and grains, often contain anti-nutritional factors and these can be inactivated by thermal processing, making nutrients such as proteins and minerals more bioavailable to the animal. For example, Duhan et al. (1989) investigated the effects of various methods of processing including soaking, cooking, and autoclaving on chickpea and black gram, and observed reduced concentrations of phytic acid, a mineral-binding compound. Van Der Poel et al (1990) found that steam processing could be used to improve the nutritive factors of beans by degrading lectins, which inhibit carbohydrate digestibility. Heat can also be used in conjunction with other treatments such as rolling or flaking of grains. For example, steam-flaking increases the digestibility of starch from corn and sorghum when consumed by dairy cows (Theurer et al., 1999). While significant research has been done looking at processing of
While heat treatment of plant-based diets such as hay may offer nutritional benefits in addition to bacterial mitigation, the nutritional integrity of animal-based diets can be negatively affected by thermal processing. Extrusion is a thermal technology commonly used to process animal-based diets for domestic carnivores (Spears & Fahey, 2004). While extrusion improves food safety and extends shelf-life and stability, this treatment may also have a negative impact on the digestibility or bioavailability of some nutrients. For the animal-based ingredients included in these diets, heat treatment can result in racemization of amino acids and Maillard reactions that result in reduced amino acid bioavailability (Carpenter, 1960; Larsen et al., 2002). Knowing the consequences of using thermal technology to treat animal-based diets, research investigating alternative and innovative treatments to mitigate bacterial contamination is warranted.

This thesis explores how processing can be used to improve diet characteristics in ways that support the nutritional integrity and palatability of a given food. In the first study, I investigated how soaking or steaming timothy-alfalfa hay affects the hay’s nutritional content and how the glycemic responses and feed preferences of Standardbred racehorses are influenced by these treatments. The second study investigated if the application of liquid methionine hydroxy analogue, Alimet® (Novus Int, Saint Charles MO, USA), an organic acid and source of methionine, can be used as an alternative to heat treatment to mitigate bacterial contamination in raw meat-based diets intended for domestic cats.
2. Literature Review for Hay-based Diets

2.1. Introduction

Dry hay is often accompanied by respirable dust particles that can be detrimental to respiratory health when inhaled (Robinson et al., 2001). To reduce respirable dust particles, moisture treatments such as soaking and steaming hay are equally effective at diminishing dust (Moore-Colyer and Fillery, 2012); however, effects of these treatments on nutrient content and bacterial contamination of hay have also been investigated, and are discussed in detail below. While the majority of research has been focused on how soaking and steaming hay may affect horses specifically, many other animals such as livestock, large exotic herbivores, and small mammalian herbivores also consume hay as an primary component of their diet (Clauss & Hatt, 2006; De Blas et al., 1999; Pasha et al., 1994) and may benefit from knowledge generated within this line of research.

2.2. Effects of processing on protein content of hay

2.2.1. Effects of soaking on protein content of hay

The effects of soaking on protein content of hay (DMB) have been varied (Longland et al., 2011; Martinson et al., 2012b) and therefore, are not well understood. One reason for this could be that protein content is estimated according to the Kjeldahl method and is therefore more accurately a measurement of nitrogen content. This does not reflect true protein content as nitrogen in plants can also be found in compounds such as phenols, tannins, alkaloids, and nucleic acids. To determine true protein content, amino acid analysis is recommended (Khanizadeh et al., 1995). Warr & Petch (1992) were the first researchers to report on how soaking hay affected the nutrient content of hay and observed crude protein losses associated
with soaking duration; however, the difference was only statistically significant when hay was soaked for 12 hours, with final crude protein constituting 9.3% of dry matter, whereas crude protein initially comprised 11.7% of dry matter (21% difference). Contrary to this, Blackman & Moore-Colyer (1998) measured losses in ryegrass, meadow, and timothy hays with an average initial crude protein value of 8.56% of dry matter, and reported the average loss of crude protein when hay was soaked for 10 minutes (7% difference) was greater than the average loss from the hays when soaked for 30 minutes (3% difference), but the difference between these losses was not statistically significant. Longland et al (2009, 2011, 2014) conducted three studies on the topic of soaking hay, creating the largest reservoir of data on the effects of soaking on nutrient content of ryegrass, timothy, and meadow hays in the United Kingdom. Their 2011 study on the effects of a 16-hour soak strongly indicated that the initial protein content of hay was the determining factor for protein changes, with greater concentrations of initial protein content (8.60% of dry matter in ryegrass) resulting in the greatest losses (5.76% of dry matter, reflecting a 33% difference). Hay with the least initial protein content (3.0% of dry matter in ryegrass-timothy mixed hay) presented an increase in protein content when soaked (3.6% of dry matter, 20% difference) and likely reflected a proportional increase in protein as other nutrients were lost (Longland et al., 2011). Martinson et al (2012b) conducted the largest study in terms of data collected on the effects of soaking time and temperature on American hays of different maturities and species, and their results were different from Longland et al (2011). They did not identify time or temperature as having any effect on crude protein content of second-cut alfalfa hay (budding stage) or first-cut orchardgrass hay (flowering stage); however in second-cut orchardgrass hay (vegetative stage) and first-cut alfalfa hay (flowering stage) soak time did significantly influence crude protein content, but with opposite effects observed in either hay.
Soaking orchardgrass hay for longer resulted in a greater proportion crude protein (DMB), but soaking alfalfa hay for longer decreased crude protein content (DMB). The researchers’ proposed explanation for these odd findings was that it is the characteristics of the nutrients themselves that may dictate how the hay is affected by soaking, with soaking duration having a mild effect on protein loss. For instance, a rise in crude protein content may simply reflect that the particular hay had a higher quantity of soluble, non-nitrogen-containing nutrients initially or that its physical structure failed to protect nutrients from being leached. On the other hand, hay with less crude protein after soaking may indicate that the hay had more soluble nitrogen. This is supported by the decreases reported in the soluble protein content of all UK meadow hays when soaked (Longland et al., 2009). Another explanation is that a decrease in crude protein content may have occurred if more protein was stored in parts of the hay which were more fragile and easily washed away as the hay was submerged or pulled out of the water. Hansen et al., (2016) examined crude protein losses from another angle, by investigating nitrogen losses in teff hay, an American grass hay known to readily accumulate nitrogen from fertilized soil. While this attribute increases crude protein concentrations, it can also become a problem when excess nitrogen is stored as nitrates, which can be toxic to horses (Oruc et al., 2010). Nitrogen concentrations in teff hay were significantly less after soaking for one hour in cold water, and even greater losses were observed when the soaking water was warm or when teff hay was soaked in cold water for eight hours. Furthermore, the nitrogen lost was nitrates, not amino acids (Hansen et al., 2016). These results suggest that soaking is an effective process for reducing nitrates to levels low enough to safely feed teff hay to horses and is also another example of how characteristics of the hay are the primary variable influencing crude protein losses, as a result of soaking, with soaking time and temperature as secondary factors to be considered.
2.2.2. Effects of steaming on protein content of hay

Unlike soaking, there is a paucity of research on the effects of steam treatment on the nutrient content of hay. Only five studies have been published regarding nutrient changes in hay as a result of steaming. Three of these studies used commercial steamers, with two using the same steamer (HG-600, Haygain Ltd., Lambourn, Berkshire, UK) (Moore-Colyer et al., 2014; Moore-Colyer et al., 2016) and the third using a different commercial steamer (The Professional Hay Steamer; Happy Horse Products Ltd., Palmyra VA, USA) (Earing et al., 2013). The others each used a system of their own design to steam hay (Blackman & Moore-Colyer, 1998; Broderick et al., 1993). All five studies measured crude protein, but none measured soluble protein (Blackman & Moore-Colyer, 1998; Broderick et al., 1993; Earing et al., 2013; Moore-Colyer et al., 2014; Moore-Colyer et al., 2016). These studies all observed either no change or a slight increase in crude protein content of steamed hay, but only one of these increases was reported to be statistically significant, with a 6% increase in crude protein contents (DMB) in UK meadow and seed hays (Moore-Colyer et al., 2016). This lack of change in the overall crude protein content of hay from steaming is expected as steaming generally conserves nutrients. As a thermal process, steaming may in fact improve bioavailability of protein in hay, as this has been well characterized in other plant-based foods (Van Der Poel et al., 1990; Rehman and Shah, 2005), although no researchers have yet investigated this in hay. In legumes, steam treatment improves protein quality by denaturing anti-nutritional factors, such as lectins, that normally interfere with the enzymatic digestion of proteins into amino acids so that they cannot be absorbed and utilized by the animal (Van Der Poel et al., 1990). The positive effect of the denaturation of anti-nutritional factors by heat is complicated by the fact that beneficial proteins can also be denatured by heat, rendering them undigestible or unavailable to the animal (Mauron,
Thermal processing can be optimized by finding the temperature and time to treat foods that balance a higher proportion of destruction of anti-nutritional factors with a lower loss of beneficial proteins (Van Der Poel et al., 1990). Therefore, investigation into the effects of steaming on amino acids and protein quality of hay and optimization of this process is warranted.

2.3. Effects of processing on carbohydrate content of hay

2.3.1. Effects of soaking on carbohydrate content of hay

Loss of carbohydrates from soaking hay has been extensively studied, as horses that consume excessive amounts of carbohydrates can be at greater risk of developing metabolic conditions such as colic, polysaccharide storage myopathy (PSSM), pituitary pars intermedia dysfunction (PPID), equine metabolic syndrome (EMS), laminitis, insulin resistance, and obesity (Borgia et al., 2009; Secombe & Lester, 2012). The most common category of carbohydrates measured are water soluble carbohydrates (WSCs) as these are the compounds readily solubilized by soaking in water and leached from hay (Blackman & Moore-Colyer, 1998; Longland et al., 2009, 2011, 2014; Martinson et al., 2012a; Müller et al., 2016; Warr & Petch, 1992), though some researchers have also measured other categories of carbohydrates, such as non-structural carbohydrates (NSCs), ethanol soluble carbohydrates (ESCs), and starch (Hansen et al., 2016; Mack et al., 2014; Watts & Sirois, 2003). Reported losses of WSCs range from as little as a 1% loss from a mix of UK hays after a 10-min soak, when initial hay WSC content was 11.94% of dry matter (Blackman and Moore-Colyer, 1998) to a 75% decrease of WSCs in first-cut American orchardgrass hay in the flowering stage (initially 13.4% of dry matter) after a 8-hour soak in cool water (Martinson et al., 2012b). This range in WSC reductions indicated that there are likely several factors involved in predicting carbohydrate loss from hay, and led to the thesis work of Wassel (2017). Wassel (2017) looked at losses of NSCs from four American hay
types at different time points from 15 minutes to 12 hours and compared this between two
different water temperatures (28°C and 50°C). Water temperature did not have a significant
effect on carbohydrate loss in any of these hays, as has been reported in other studies when
looking at a similar comparison of water temperatures (22°C and 39°C) (Martinson et al.,
2012b), and similar to protein, suggests that differences in the characteristics of the hay itself
may be one of the most important factors to consider for optimal hay soaking. Future research
should investigate how soaking affects the carbohydrate content of each hay species individually.

2.3.2. Effects of steaming on carbohydrate content of hay

Three studies in the UK (Blackman and Moore-Colyer, 1998; Moore-Colyer et al., 2014;
Moore-Colyer et al., 2016) and one in the US (Earing et al., 2013) have reported on carbohydrate
content changes in hay as a result of steaming. The work of Earing et al (2013) revealed that a
90-min steam decreased WSCs by 12% in mouldy hay (initially 9.37% of dry matter), while hay
with low mould (initially 9.63% of dry matter) did not result in the loss of WSCs, even though
initial WSC content was similar for the two hays. Earing et al (2013) believed this to have
occurred because the larger quantities of mould had formed in the moderately mouldy hay as a
result of more moisture being present at the time of baling. This greater moisture in the hay could
have solubilized more carbohydrates than in the low mould hay prior to steaming, then during
the steaming process the solubilized carbohydrates were more readily leached away. The only
other significant change observed in WSCs by steaming was an 18% decrease in meadow and
seed hays (initially 12.6% WSC on a DMB) after a 50-min steam (Moore-Colyer et al., 2016),
which was quite surprising since in their preceding study, hays with identical levels of WSCs had
been steamed in the same steamer for 40 minutes and observed a 3% decrease of WSCs (Moore-
Colyer et al., 2014). Moore-Colyer et al (2016) were unsure of an explanation for this and point
out the larger unexplained variation in WSC losses from soaking. However, it had been discussed earlier in the paper that the hays sampled in that study could be categorized as moderately mouldy, which may provide sufficient justification to further investigate the theory of Earing et al (2013) that moisture content at the time of baling may result in greater soluble carbohydrate losses from steaming.

2.3.3. Effects of carbohydrate content on glycemic response in horses

In horses, the WSC content of a meal is an important factor in predicting the glycemic response, the rise and fall of blood glucose levels from consuming a meal, as this group of nutrients mostly consists of sugars that are readily solubilized and rapidly absorbed in the small intestine (Gordon et al., 2007; Borgia et al., 2009). To date however, only two studies have been conducted to compare glycemic response in horses from consuming dry and soaked hay. In the first study, two batches of chopped orchardgrass hay were soaked for 30min for two glycemic response trials using four- to five-month-old weanling fillies (Cottrell et al., 2005). In the first trial with these horses, soluble sugars were reduced from 12% to 5.6% DM in hay, and all horses were fed the same quantity of hay on a DMB (0.34 – 0.48% of each horse’s body weight (BW) on a DMB). This resulted in no difference between peak glucose values, but the area under the curve (AUC) was smaller in horses fed soaked hay compared to horses fed dry. In the second trial, soluble sugars were reduced from 22% to 13.4% DM, and all horses were fed 0.3% BW on a DMB. The peak glucose value was lower and the AUC was smaller in horses fed soaked hay in comparison to horses fed dry. Additionally, the glycemic response from dry hay in the first trial was similar to that of soaked hay in the second trial, which appears to support the hypothesis that the glycemic response in horses to hay can be predicted based on soluble sugar content as this was similar between the two hays. The other study, Collins (2015) used mature adult horses
(between 13 and 21 years of age), two hay types: prairiegrass and alfalfa, and each horse was fed 0.5% BW on a DMB. Hay was soaked in cold water for 60 minutes, which reduced water soluble carbohydrates in prairiegrass hay from 9.5% to 6.8% DM and in alfalfa hay from 8.8% to 6.6% DM (statistical significance not reported). No differences were found between glycemic response variables from horses fed soaked and dry hay. These contrasting results from the two studies indicate that further investigation into the effects of soaking hay on the glycemic response is needed. In addition, the glycemic response from consuming steamed hay has never been studied and given that some studies have reported statistically significant changes in WSC content from steaming (Earing et al., 2013; Moore-Colyer et al., 2016), this also warrants investigation.

2.4. Effects of processing on mineral content of hay

2.4.1. Effects of soaking on mineral content of hay

Moore-Colyer (1996) investigated if soaking affected mineral content of hay for horses, specifically sodium, potassium, phosphorus, calcium, and magnesium. Soaking resulted in significant losses of sodium, phosphorus, and potassium when hay was soaked for 30 minutes, and a significant loss of magnesium when soaking time was increased to three hours. Since sodium and potassium play important roles in muscle contraction, losses of these nutrients could result in reduced electrolyte status and negatively impact equine athletic performance, especially since horse sweat is hypertonic, resulting in large losses of electrolytes in their sweat (Ecker and Lindinger, 1995). Blackman & Moore-Colyer (1998) further investigated mineral losses from short soaking periods and examined the same minerals as Moore-Colyer (1996), as well as iron, copper, zinc, and manganese. Blackman & Moore-Colyer (1998) reported significant losses of potassium, magnesium, and copper from a 10-min soak, and significant losses of sodium after a 30-min soak. They noted considerable variation in mineral content between different hay
samples, which they speculated may explain some of the variation in mineral losses (Blackman & Moore-Colyer, 1998). The loss of minerals from soaking hay is likely to be perceived as a negative effect by many horse owners and managers, not just for athletic horses as, if hay is the only source of nutrients for a given horse, over-soaking could result in hay that no longer meets that horse’s micronutrient needs when consumed to dry matter requirements. Following the work of Blackman & Moore-Colyer (1998), Watts & Sirois (2003) assessed carbohydrate and potassium concentrations in a large variety of American hays following a 30-min soak in hot or room temperature water, or a 60-min soak in room temperature water with the intent of lowering potassium levels to less than 1.1% of dry matter, which is the concentration that has been deemed safe for horses at risk of developing hyperkalemic periodic paralysis (HYPP), a rare but serious condition (Reynolds et al., 1998). For the most part, the 30-min soak in hot water and the 60-min soak in room temperature water lowered the potassium in the hays to less than 1.1% of dry matter, but there were some exceptions that did not appear to be dependent on initial potassium content. Hansen et al (2016), who studied teff hay with a potassium concentration of 3% of dry matter, observed a decrease to less than 1% of dry matter when hay was soaked for 60 minutes in cold and warm water, as well as when it was soaked for 12 hours in cold water. Similar to Moore-Colyer and Blackman’s findings (1996; 1998), large decreases in phosphorus and sodium content were observed when hay was soaked under these three conditions. Additionally, losses of zinc were reported when hay was soaked for eight hours. While the losses of both potassium and sodium may put performance horses at risk of deficiency, the loss of phosphorus is equally concerning. This is because many compounds found in plants will bind phosphorus, making it unavailable to the animal consuming it (van Doorn et al., 2004). Furthermore, it is likely that the phosphorus lost during soaking is unbound phosphorus and the
phosphorus left behind is less bioavailable. Unlike Hansen et al (2016), Martinson et al (2012b) only observed potassium levels decrease to less than 1.1% of dry matter in any hay after a 12-hour soak. Calcium losses appeared to be primarily related to forage maturity, with significant losses from second-cut hays, but not first-cut, even though calcium levels were similar between the two different maturities within each hay species, but not similar between species at any stage (Martinson et al., 2012a). Phosphorus was significantly reduced in three out of four hays (first- and second-cut orchardgrass and first-cut alfalfa) after a 15-min soak in cold water, and the loss was significant in the fourth hay (second-cut alfalfa) after a 30-min soak in cold water, with losses increasing with soaking time. Magnesium was lost from both cuts of alfalfa hay, in the 15-min cold soak, but losses were only statistically significant in second-cut orchardgrass in the 15-min warm soak, and in first-cut orchardgrass in the 30-min warm soak, but not the 30-min cold soak. Finally, Mack et al (2014) also observed potassium concentrations reduced to less than 1% of dry matter when hay was soaked for more than eight hours. They are also the only researchers to have measured chloride and sulfur in soaked hay, both of which were significantly lower after a minimum of eight hours of soaking, along with calcium, phosphorus, magnesium, and sodium. Overall, the variation in losses reported across soaking studies suggests that mineral leaching from hay depends on a number of factors such as characteristics of the hay itself, soaking time and temperature, and mineral solubility. In the case of potassium, there is no evidence to support soaking times less than eight hours as sufficient to reduce to concentrations considered safe for HYPP horses. These longer soaking times will increase other mineral losses and may result in dietary supplementation being required. Since most horses are not at risk of HYPP and do not require a low potassium diet, mineral losses are generally viewed as a negative effect of soaking and could be detrimental to horses whose diet solely consists of hay.
2.4.2. Effects of steaming on mineral content of hay

Studies on changes in mineral content before and after steaming have generally reported few differences. The calcium content of steamed hay was mostly unchanged in the three studies that measured it (Blackman and Moore-Colyer, 1998; Earing et al., 2013; Moore-Colyer et al., 2016). Hay with high initial levels of phosphorus (0.34% and 0.32% of dry matter) had a decrease in phosphorus (15% and 16%, respectively) when steamed for 90 minutes (Earing et al., 2013). Hays with lower initial phosphorus levels (0.196% and 0.1% of dry matter) increased when steamed for 80 and 50 minutes, respectively (39% and 100% differences, respectively) (Blackman and Moore-Colyer, 1998; Moore-Colyer et al., 2016), which may indicate that initial phosphorus level is a key factor in determining phosphorus changes from steaming. For other minerals, one study reported changes in potassium were significant, but only 0.1% higher in steamed hay compared to dry hay and likely not biologically relevant (Moore-Colyer et al., 2016). Another study reported no differences in the potassium content of steamed and dry hay (Blackman and Moore-Colyer, 1998). Finally, small differences in sodium, magnesium, copper, and manganese were not significant (Blackman and Moore-Colyer, 1998; Moore-Colyer et al., 2016), but the 7% increase in zinc found by Moore-Colyer et al (2016) was significant. Overall, the lack of agreement on the effects of steaming on hay mineral content suggests more research is warranted and a robust understanding of variables such as species, maturity, time stored, and other nutrient contents needs to be considered.

2.5. Effects of processing on vitamin and vitamin precursor content of hay

To date, no studies have assessed how soaking or steaming affect vitamins or their precursors in hay. Given the water-soluble nature of the B vitamins (thiamin, riboflavin, pantothenic acid, nicotinic acid, pyridoxine, folic acid, and biotin) it would be expected that
soaking and steaming would leach these nutrients to a similar extent as the water-soluble carbohydrates. However, it is unknown how this would affect the horse, as generally, the B vitamins needed by the horse are synthesized in the cecum and hindgut (Carroll et al., 1949).

2.6. Horse preferences for hay when steamed or soaked

In addition to determining the effects of steaming and soaking on characteristics of the hay itself, it is also paramount to determine if horses find hay palatable when it is processed in these ways and to investigate if either of these treatments are preferred by horses in comparison to each other and their normal dry hay. The change in nutrient content is an important factor that could impact a horse’s response to soaked and steamed hay. Researchers have indicated that horses have a preference for sweet tastes (Danel and Merkies, 2009; Merkies and Bogart, 2013). The two studies which have investigated horse preferences for dry, steamed, and soaked hay so far have taken two different approaches that have led to similar but inconclusive results. The first study was conducted as a three-way comparison in which dry, steamed, and soaked hay were offered together to six Polo ponies for 60 min at three different feedings (Moore-Colyer and Payne, 2012). Steamed hay was consumed in the greatest quantity and soaked hay constituted the lowest quantity consumed on a DMB. In contrast, Pagan et al. (2013) offered each hay separately for four week periods to three Thoroughbred geldings. Daily intake was greater for steamed hay compared to soaked, while dry hay did not differ from either steamed or soaked. While these findings suggest steamed hay may be preferred by horses over soaked hay, both of these studies had very low sample sizes (n=6 and n=3, respectively), making it difficult to have confidence in these results. More research with a larger sample size is warranted.
2.7. Effects of processing on bacterial content of hay

2.7.1 Effects of soaking on bacterial content of hay

Since water activity is a key determinant in predicting bacterial growth (Davey, 1989), it stands to reason that adding moisture to dry hay would raise concerns of increasing bacteria colony counts in hay. Based on the early work of Moore-Colyer et al (2012; 2014) these concerns were validated when a 10-min soak increased total viable counts by 1.7-fold and a 9-hour soak increased colony counts by 5.9-fold. In contrast to this, Müller et al (2015) did not observe a difference in enterobacteria levels when a mix of timothy, meadow fescue, and red clover hay was soaked in cold tap water for 24 hours; however, they also conducted this soaking experiment with silage and haylage that had lower initial contamination levels than the hay and enterobacteria did increase in these after soaking. This suggests that initial contamination level may be an important factor in predicting changes to bacteria content from soaking. This is supported by the recent work of Moore-Colyer et al (2018), who investigated the effects of soaking on hay with higher total viable counts of bacteria and did not observe differences in colony counts between dry hay and hay soaked for 90 min, 9 hours, or 16 hours. Moore-Colyer et al (2018) were also the first researchers to attempt to characterize these changes in bacteria based on genomic classification; however, these changes again appeared to be closely associated with initial bacterial profiles of the hays.

2.7.2. Effects of steaming on bacterial content of hay

In contrast to soaking, the heat treatment of steaming reduces bacteria in hay. James & Moore-Colyer (2010) were the first to demonstrate this by steaming bales of ryegrass meadow mix hay for 50 minutes in a Haygain HG-2000 steamer (Haygain Ltd., Lambourn, Berkshire,
UK), which resulted in a 98.8% reduction in the total viable counts of bacteria in hay, from $3.8 \times 10^5 \text{ CFU/g}$ to $4.4 \times 10^3 \text{ CFU/g}$. This was followed up by Moore-Colyer & Fillery (2012) who steamed half-bales of ryegrass hay for 40 minutes in a Haygain HG-600 steamer (Haygain Ltd., Lambourn, Berkshire, UK), and observed a 99.6% decrease in total viable counts, from $2.18 \times 10^7 \text{ CFU/g}$ to $8.0 \times 10^4 \text{ CFU/g}$ (DMB). Moore-Colyer et al (2014) repeated this second steaming experiment with five different seed and meadow hays with a much lower initial total viable count ($6.0 \times 10^5 \text{ CFU/g DM}$) and from this measured a 98.2% decrease of bacteria ($1.0 \times 10^3 \text{ CFU/g DM}$). Finally, using the same model of steamer, Moore-Colyer et al (2016) increased steaming time to 90 minutes, and total viable counts in meadow hay were reduced from $2.3 \times 10^5 \text{ CFU/g DM}$ to $12 \text{ CFU/g DM}$ (99.99%). Since these studies demonstrated that steaming decreased bacterial concentrations by over 98% in all cases, steaming is clearly an effective process for reducing bacterial contamination in hay.

2.8. Conclusions

In general, nutrient losses from soaking hay appear to be heavily dependent on how fragile the hay is and how easily its nutrients are solubilized, and these losses will be exacerbated by longer soaking times and higher water temperatures. In comparison to soaking, steaming generally conserves nutrients; however, the impact of heat on the structure and function of nutrients in hay has not yet been sufficiently studied and future research is warranted in this area. Studies on how soaking impacts the glycemic response in horses have reported conflicting results and no studies have investigated how steaming affects this. Research into horse preferences has not been conclusive but suggests that horses prefer steamed hay over soaked hay. In terms of bacterial contamination, the process of soaking appears to increase bacterial contamination in hay with low initial levels of bacteria; however, these levels remain relatively
unchanged if hay is already highly contaminated. Steaming, on the other hand, vastly improves hygienic quality of hay, regardless of initial contamination level and longer steaming time may result in greater reductions of bacteria.
3. Literature Review for Meat-based Diets

3.1. Introduction

Bacterial contamination is one of the biggest concerns surrounding the provision of meat-based diets for animals (CVMA, 2018). While thermal technologies are quite effective at alleviating bacterial contamination, they can also damage nutritional components of these diets such as amino acids (Tran et al., 2008). A small but significant, consumer trend in pet foods has recently shifted towards feeding uncooked, raw meat-based diets (RMBDs) (Lange, 2016) and this interest is partly supported by suggestions of nutrient losses in cooked food and improved functional nutrient delivery in raw food. In addition to feeding raw diets to dogs and cats, carnivores in zoos and aquariums are typically fed raw meat-based diets (Kerr, 2012) and these diets also often present high bacteria loads (Lewis et al., 2002). With the increased interest in raw foods the need to investigate alternative methods to heat treatment to mitigate bacterial contamination in RMBDs is warranted. In human nutrition, one practice that is commonly used when looking to avoid thermally processed animal products, is the application of organic acids (Theron and Lues, 2007).

3.2. Microbes in meat

While animal muscle tissue is generally considered sterile pre-slaughter, once animals are slaughtered, muscle tissue is commonly exposed to bacteria found in their intestinal tract, their own exterior, or the equipment used, and as a result the surface of the meat becomes contaminated (Elder et al., 2000; Buncic and Sofos, 2012). While not all microbes are harmful, the bacteria of consequence are those that are pathogenic, cause spoilage, or both (Vlahova-Vangelove and Dragoev, 2014). In Canada, the bacteria that have been most commonly
associated with foodborne illnesses in humans are *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, and Salmonella* spp. (PHAC, 2016).

When identifying bacteria, one of the most defining features is the outer protective layer of the cell, and it is hypothesized that this feature is the determining factor of the Gram stain, named after its discoverer Christian Gram (Gram, 1884; Bartholomew and Mittwer, 1952). Typically, microbes with a thick cell wall surrounding an inner cell membrane will stain purple and are called Gram-positive bacteria, and those with a thinner structure that is referred to as a cell envelope surrounding the inner cell membrane will stain red and are referred to as Gram-negative bacteria (Salton and Kim, 1996). This distinction between outer cell structures is important as it is thought to be one of the key factors in how these groups of bacteria respond to environmental stress differently (Jordan et al., 2008; Ramos et al., 2001).

### 3.3. Organic acids as antimicrobials

The application of organic acids could be considered one of the most diverse technologies for meat preservation and pathogen mitigation, as it may be employed as early as immediately following slaughter in the form of a carcass wash (Huffman, 2002) and as late as minutes before consumption, such as a condiment used on raw meatballs (Bingol et al., 2011). Organic acids are generally well-accepted by the public, as they naturally exist in foods and have a long history of use for food preservation (Ricke, 2003). Organic acids are hypothesized to function by diffusing into the cell as protonated molecules that then dissociate until the buffering capacity of the cell is overwhelmed and the pH of the formerly neutral cytoplasm is lowered and becomes acidic, and cell function is inhibited (Carpenter and Broadbent, 2009). With consideration for the bacteria most commonly suspected as the culprits of foodborne illness in Canada, *Salmonella* spp. and *Listeria monocytogenes* are the pathogens that have also been most
frequently identified in meat-based diets for pets sold in Canada and the United States by the FDA. In 2018, 21 of the 22 raw pet products recalled by the FDA were recalled due to concerns or confirmation of the presence of *Salmonella* spp., *Listeria monocytogenes*, or both (FDA, 2018).

### 3.3.1. Effectiveness of organic acids against *Salmonella* spp.

*Salmonella* is a genus of Gram-negative, rod-shaped bacteria (El-Gazzar and Marth, 1992) that is recognized as having two species, *Salmonella enterica* (formerly *Salmonella choleraesius*) and *Salmonella bongori*. Within the *Salmonella enterica* spp., `enterica` is usually dropped from the name when microbes can be identified by serovar and strain (Su and Chiu, 2006). *Salmonella* spp. growth and survival depends on a number of factors including which serovar is being tested, and environmental conditions such as temperature and pH (Gibson et al., 1988). The lowest pH that a *Salmonella* spp. has been observed to continue to grow at was 4.05 when the environment was acidified with hydrochloric or citric acid, and higher pHs when other acids were used (Chung and Goepfert, 1970). Organic acid testing against *Salmonella* spp. survival in meat environments is typically conducted using *S. Typhimurium*. The most widely utilized organic acids in carcass decontamination that are effective against *S. Typhimurium* are solutions of 0.5 – 2.0% acetic, lactic, and citric acids which result in 0.8 – 2.5 log CFU reductions (Buncic and Sofos, 2012). When comparing fumaric acid to lactic and acetic acid, 1.0% fumaric acid was found to be more effective than both lactic and acetic acid at inhibiting *S. Typhimurium*, and 1.5% fumaric acid was more effective than 1.0% (Podolak et al., 1995).
3.3.2. Effectiveness of organic acids against *Listeria monocytogenes*

*Listeria monocytogenes* is a species of Gram-positive, rod-shaped bacteria that grows best in aerobic environments between 30°C and 37°C (Low and Donachie, 1997); however it can still survive and grow at temperatures as low as 2°C, which is problematic in a foodborne pathogen because it can continue to multiply under refrigeration conditions (Gandhi and Chikindas, 2007). While growth of *L. monocytogenes* can be hindered by a mildly acidic environment (pH 5.6), this species also carries many tools for adapting to survive acidic conditions (Cotter and Hill, 2003). When comparing acetic, lactic, and fumaric acids, fumaric acid was found to be most effective at inhibiting *L. monocytogenes*, and at 2.0%, resulted in a 0.94 log unit reduction (Podolak et al., 1996).

3.4. Methionine hydroxy analogue: An existing supplement as an antimicrobial

A common practice in complete foods is to improve existing diets rather than creating an entirely new formula. It is even more appealing to improve a formula if a new ingredient has a duplicitous role, such as acting as an antimicrobial and providing an essential nutrient, such as an amino acid (Dibner and Buttin, 2002). Methionine hydroxy analogue (MHA) is a methionine supplement used as an alternative to DL-methionine (DLM). Methionine supplements are used to improve protein quality especially in diets containing meat and poultry as methionine is considered the first limiting amino acid in animal-based diets (Klemesrud et al., 1997).

The structure of MHA differs from DLM in that it bears a hydroxyl group (OH) in place of the amine group (NH₂) (Novus Int, 2017). Work in poultry has established that MHA is absorbed primarily by passive diffusion, while DLM relies mainly on carrier-specific transport systems (Dibner et al., 1992). The majority of the research suggests that MHA is less
bioavailable than DLM in monogastrics based on research in poultry, swine, and fish (Powell, Chowdhury, & Bureau, 2017; Rychen et al., 2018; Sauer et al., 2008; Shoveller et al., 2010).

The chemical name of MHA is 2-hydroxy-4-(methylthio) butanoic acid and it is similar in structure to fumaric acid. It is available in liquid form as Alimet® (Novus Int, Saint Charles MO, USA) and has a molecular weight of 149.00 and a pKa of 3.86 (Dibner and Buttin, 2002). Fumaric acid has been shown to be a more effective antimicrobial against *E. coli, L. monocytogenes, and S. Typhimurium* when tested as a 1% dip solution for lean beef tissue, when compared to 1% lactic acid and 1% acetic acid (Podolak et al., 1995a). When compared to organic acids already commonly tested in swine and poultry feeds, MHA was completely bactericidal against *E. coli* after 24 hours and was equally effective as formic acid when each was included as 1% of tryptic soy broth and pH was adjusted to 4.0 using HCl and NaOH (Dibner and Buttin, 2002). Given the similarity in structure between MHA and fumaric acid, and the performance of MHA against *E. coli*, MHA would be expected to work effectively against *L. monocytogenes* and *S. Typhimurium*. For the purposes of animal-based raw diets, the addition of MHA could provide a bactericidal role in addition to its nutritional benefits, and this warrants investigation.

### 3.5. Conclusions

The use of organic acids to mitigate bacterial contamination in raw meat products is an accepted effective practice that is dependent on target pathogen susceptibility to acids as well as which particular acid and strength are used. Fumaric acid appears to more effectively inhibit both *S. Typhimurium* and *L. monocytogenes* than lactic or acetic acid of equal concentration. MHA is similar in structure to fumaric acid and has inhibited growth of *E. coli* in nutrient broth, in addition to improving protein quality of diets. Investigation into the effectiveness and safety of
using MHA to mitigate bacteria in raw pet diets may prove to be an acceptable alternative to heat treating pet food.
4. Research Rationale and Objectives

The improvement of current animal diets through simple changes to formulation and preparation is a feasible practice for animal feed producers and animal owners. Steaming hay appears to be an effective means of reducing dust and bacteria while generally conserving nutrients, while soaking can leach nutrients and increase bacteria counts. There may be some benefits to reducing certain nutrients in hay such as carbohydrates. As such, the objectives of my first thesis study were to characterize nutrient changes in Ontario-grown hay from soaking and steaming, and to assess if these changes impacted the glycemic response and preferences of Standardbred racehorses. I hypothesized that soaked hay would have decreased carbohydrates that would illicit a lower glycemic response and be less preferred in comparison to normal dry hay, and that steaming would do this to a lesser extent than soaking.

Methionine supplementation is commonly employed to improve protein quality of animal diets. Methionine hydroxy analogue (Alimet®) as an organic acid may function as an antimicrobial that could be included in raw pet diets to mitigate bacterial contamination. The aim of my second thesis study was to establish the minimum concentration of Alimet® needed to eliminate Salmonella spp. and Listeria monocytogenes from raw ground meat, as would be used to formulate raw pet diets. I hypothesized that Alimet® included as 1% of the sample would effectively eliminate these pathogenic bacteria in raw meat.
5. Nutrient content changes from steaming or soaking timothy-alfalfa hay: Effects on feed preferences and acute glycemic response in Standardbred racehorses

5.1. Abstract

Soaking hay and steaming hay are strategies that are used to reduce respirable dust particles for horses, but may result in variable nutrient losses, including water-soluble carbohydrates (WSC) and minerals. Since these losses have not been quantified in Canadian hay yet, the first aim of this study was to identify nutrient losses from first-cut timothy-alfalfa hay grown in southern Ontario, Canada after soaking for 30 min or steaming for 60 min. It is uncertain whether horses prefer hay when it is dry, soaked, or steamed. To address this, 14 Standardbred racehorses were offered two of these hays side by side for 30 min on six consecutive occasions until all possible combinations had been offered. Quantity of hay eaten was determined and horses were video recorded during feedings to assess time spent eating and investigating hay. Additionally, consumption of feeds with differing WSC levels has been observed to influence glycemic response in horses; however, this has not been measured in horses consuming steamed hay before and the results from soaked hay studies have been inconclusive, therefore, the final aim of this study was to examine acute glycemic response in horses after being fed dry, soaked, and steamed hays. Blood glucose was measured every 30 min from nine Standardbred racehorses for 6 h following a meal of 0.5 % BW of treatment hay. Soaked, but not steamed hay, had lower concentrations of WSC, soluble protein, and potassium in contrast to the same dry hay (P<0.05). Peak glucose, average blood glucose, total area under the curve (AUC), and time to peak did not differ among treatments (P>0.05). We conclude that acute glycemic response of racehorses was not influenced by soaking or steaming hay. Horses
also consumed less soaked hay on a dry matter-basis than dry or steamed hay (P>0.05), and spent less time eating soaked hay when dry hay was the alternative (P>0.05).

5.2. Introduction

Soaking and steaming hay are two processes that are recommended for feeding horses primarily to reduce respirable dust particles. Soaking hay is a more commonly used method, as it does not require any specialized equipment, and has been shown to reduce respirable dust particles as effectively as steaming hay (Blackman and Moore-Colyer, 1998; Moore-Colyer and Fillery, 2012). Steaming hay, using a commercial steamer has been shown to decrease mold by 96.4 - 99.9 % and can also lower bacteria colony counts by 93.0 - 99.7%, as opposed to soaking hay that can increase bacteria by 1.5 – 4 fold depending on initial bacterial load (Moore-Colyer & Fillery, 2012; Moore-Colyer, Lumbis et al., 2014).

Nutrient losses in hay from soaking appear to be variable and are dependent on a number of factors including type and maturity of hay, temperature of soaking water, and amount of time hay is processed for. Nutrients lost when soaking hay may include: phosphorus, potassium, magnesium, sodium, copper, calcium, protein, water-soluble carbohydrates (WSC), and non-structural carbohydrates (NSC) (Blackman & Moore-Colyer, 1998; Hansen et al., 2016; Longland et al., 2009, 2011, 2014; Mack et al., 2014; Martinson et al., 2012a, 2012b; Moore-Colyer, 1996; Moore-Colyer et al., 2014; Müller et al., 2016; Warr & Petch, 1992; Watts & Sirois, 2003). Steaming hay generally conserves nutrients and may increase the availability of some nutrients such as protein, potassium, and zinc, however, losses of phosphorus and WSC have also been reported, though the degree of nutrient loss is much lower than soaking (Blackman & Moore-Colyer, 1998; Broderick et al., 1993; Earing et al., 2013; Moore-Colyer et al., 2014; Moore-Colyer et al., 2016).
Changes in nutrient content of foodstuffs can affect how food tastes and is one important factor in food selection by animals (van den Berg et al., 2016). Understanding equine food preferences can help owners and managers determine how to effectively provide nutrients to the animals. Soaking hay is expected to alter hay taste by removing nutrients and will likely also change the texture of the hay due to the substantial absorption of water. Since horses prefer sweet tastes, the loss of carbohydrates from soaking hay may decrease its palatability (Danel and Merkies, 2009; Merkies and Bogart, 2013). Furthermore, it is unknown how horses will perceive a change in texture that will occur by soaking. Steaming, on the other hand, is expected to influence palatability as the application of heat and steam may result in the formation of different volatile compounds which will alter the odour (Picardi and Issenberg, 1973). The taste and texture of hay is expected to be minimally affected by steaming since steaming generally conserves nutrients (Earing et al., 2013; Moore-Colyer et al., 2014; Moore-Colyer et al., 2016), and only one study has ever observed an increase in moisture from steaming (Earing et al., 2013).

Non-structural carbohydrate (NSC) content of hay, including WSC, ethanol-soluble carbohydrates (ESC), and starch, is a common subject of interest in equine nutrition research as NSCs are correlated with differences in glycemic and insulinemic response in horses (Borgia et al., 2009; Gordon et al., 2007; McGowan et al., 2013). Hay with high NSC content may not be suitable for horses at risk of developing or that have been diagnosed with insulin-resistance and conditions associated with it (Borgia et al., 2009; Secombe and Lester, 2012). It is recommended that hay for these horses should consist of 10% NSCs or less, and since carbohydrate losses from soaking can be variable, that soaking should only be used to reduce hay NSCs to this level if the initial content level is below 12% (Borgia et al., 2009; Frank et al., 2010). Despite this existing
recommendation, studies of the acute glycemic response of horses from consuming soaked hay have demonstrated conflicting results (Collins, 2015; Cottrell et al., 2005) and this has never been studied using steamed hay.

Previous research on nutrient losses in hay have been conducted using hays grown in the UK, the US, and in Sweden (Warr and Petch, 1992; Broderick et al., 1993; Moore-Colyer, 1996; Blackman and Moore-Colyer, 1998; Watts and Sirois, 2003; Longland et al., 2009; Longland et al., 2011; Martinson et al., 2012b; Earing et al., 2013; Longland et al., 2014; Mack et al., 2014; Moore-Colyer et al., 2014; Hansen et al., 2016; Moore-Colyer et al., 2016; Müller et al., 2016), and nutrient losses in Ontario-grown hay will likely vary due to characteristics of different hay species and growing conditions. Mixed timothy and alfalfa hay is one of the most common hays fed to horses in Ontario (Wright, 1999). Alfalfa hay is a legume hay that is relatively high in crude protein and calcium, while timothy is a grass hay and relatively low in crude protein and may not meet horse protein requirements alone (Cuddeford et al., 1992; Woodward et al., 2011; Yu et al., 2003). Providing these hays as a combination, especially when grown together as they are in Ontario, generally offers a more moderate level of crude protein overall (Ta and Faris, 1988).

The primary goal of this study was to assess the impact of soaking and steaming on nutrient content of first-cut Ontario-grown mixed timothy-alfalfa hay. In addition, while some researchers have made observational comments regarding the unequal distribution of moisture through hay from soaking or steaming, no research has reported if this has affected nutrient losses throughout hay. As such, this study also sought to investigate if nutrient content differed within the treated hay, as well as among hay treatments. We also sought to determine if soaking or steaming hay impacts the acute glycemic response in horses and whether these horses
demonstrate a preference for hay when steamed, soaked, or dry. We hypothesized that soaking hay would result in decreased NSC,WSC, phosphorus, potassium, magnesium, copper, calcium, and sodium, and that steamed hay would have lower reductions of WSC and phosphorus while conserving all other nutrients. We also hypothesized that the decreases in carbohydrates in soaked and steamed hay fed to horses would correspondingly reduce their preference for these hays, and also that carbohydrate losses would lead to correspondingly lower glycemic responses in contrast to natural dry hay.

5.3. Materials and methods

All experiments and procedures involving animals were approved by the University of Guelph Animal Care Committee (AUP #3783) and performed in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

5.3.1. Hay nutrient analyses

A total of 39 hay samples were collected for nutrient analyses. Two different steaming protocols and two different soaking protocols were employed in this study; one protocol was used for each treatment for preparing hay twice daily for the feeding experience study (“daily protocol”) and one protocol was used for each treatment for preparing hay the night before the glycemic response tests (“overnight protocol”). Details of the preparation of these treatments and sample collection are described in the corresponding trial section. Triplicate samples of each treatment were collected during trials to compare the effect of these differing protocols, resulting in 12 samples in total from the trials. All other samples were prepared and collected as described below.
All hay used in the study was first-cut mixed timothy-alfalfa hay, grown in southwestern Ontario on, and supplied by, the same farm the participating horses were stabled at.

**Hay treatments**

A single small bale of hay was randomly selected from a stack to compare the effects of the three treatments (dry, steamed, or soaked) as prepared using the “daily protocol”. The 17.5kg bale was partitioned and subjected to the following treatments: two flakes (~1.59 kg, as-is) were left dry (untreated), two flakes were placed into separate hay nets to be soaked, and the remainder of the bale was steamed. The two flakes in the hay nets were immersed in room temperature tap water (25°C) in a 90L clean plastic bin. After soaking for 30 minutes, the hay nets were then hung to drain for 30 minutes. The remainder of the bale was steamed for 60 minutes in the commercial hay steamer (HG-2000; Haygain Ltd, Lambourn, Berkshire, UK) according to the manufacturer’s instructions, such that the internal temperature of the steamer chest was between 74°C and 85°C for the final 10 minutes of steaming, then extracted from the steamer chest and left to cool on a tarp for 30 minutes.

**Hay sample collection**

Two discrete sets of samples were collected from the hay described above. The first set was collected to assess differences in nutrient content between the three hay treatments. Three random samples were collected from each set of treated hay flakes. To get a representative sample, a grab sample of hay was collected in a W-pattern, resulting in three “all over” samples for each treatment, and nine samples in total for this set.
The second set of samples was collected to assess for differences within the soaked and steamed treatments, as it was unknown if the treatments were affecting the hay evenly throughout. Three random samples were collected from each given area (top, middle, and bottom) relative to how the hay was placed in the soaker or steamer. This was done by separating the treated hay into three sections (top, middle, and bottom) and collecting grab samples in a W-pattern from each section. Three samples were collected for each section, resulting in three top, three middle, and three bottom samples for each treatment, and 18 samples in total for this set.

For all collections, samples were collected and placed into sterile plastic bags and were kept at room temperature (20°C) and submitted for nutrient analyses, which were performed within one hour of collection, as described below.

**Nutrient analyses**

The hay samples were submitted to the Agri-Foods Laboratories (Guelph, ON) for analysis. Each sample was dried and milled before being separated into subsamples for different components of the analysis. Dry matter and moisture were determined according to Goering & Van Soest (Goering and Van Soest, 1970) by the oven drying method. The total ash and individual minerals, calcium, phosphorus, potassium, magnesium, sodium, zinc, copper, iron, and manganese were analyzed by inductively coupled plasma as outlined by the AOAC (AOAC, 1996). Crude protein (CP), soluble protein (SP), a-neutral detergent fibre (aNDF; the a stands for α-amylase, which was used to remove starch and protein), acid detergent fibre (ADF), lignin, fat, water soluble carbohydrates (WSC), non-structural carbohydrates (NSC), ethanol soluble carbohydrates (ESC), and starch were determined using near-infrared reflective (NIR)
spectroscopy according to the manufacturer’s instructions (Spectra Star RTW, Unity Scientific, Milford MA, USA; Software: InfoStar V3.11.1)).

**Tap water collection and mineral analyses**

To account for minerals that may have been introduced to hay from soaking water, three 250mL samples of tap water used for soaking were submitted to the Agri-Food Laboratories (Guelph, ON) for mineral analyses. From these samples, phosphorus, potassium, calcium, magnesium, sodium, iron, zinc, manganese, and copper were analyzed by inductively coupled plasma – atomic emission spectrometry as outlined by the United States Environmental Protection Agency in Method 6010D (U.S. EPA., 2014).

**Calculation of tap water contribution**

The fraction of each respective mineral that was present in the tap water was multiplied by the proportion of moisture in the soaked hay (assuming at best that all of the previous moisture in the hay had been replaced by the tap water). This value was divided by the total fraction of each mineral that was present in the soaked hay on an as-is basis. This gave the highest possible proportion of each mineral that could have been introduced into the hay by the tap water.

**5.3.2. Feeding experience study**

**Horses**

Fourteen healthy, privately-owned, Standardbred racehorses (457 ± 45 kg, mean ± SD; 5 geldings, 9 mares; 1.5 – 9 years of age) were selected for the feeding experience study to assess
preferences for hay when offered dry, steamed, or soaked. Horses were stabled in the same barn and managed by the same team of trainers. Horses were consciously allocated into three cohorts based on trainer requests that pertained to racing schedules. One horse was on stall rest for the duration of the study; all other horses trained six days a week. Each horse was kept in a 3 × 3 m box stall, built with wood planks. At the front of the stall horses could freely stick their heads out to look out over the stall door, which was on the left-hand side when looking at the stall from the outside. On the right-hand side was a wall with a metal grill at the top to allow horses to see out, but not stick their head through. Just below the bars two buckets were secured to the wall; the bucket near the corner was for grain and the other bucket was filled with fresh water daily. A salt block was hung on the right-side wall so that horses had free access to salt. Each horse was stabled in the same stall throughout the study and stalls were bedded with fresh wood shavings daily. Prior to the beginning of the study, horses were being given hay which was lightly hosed off and drained in a wheelbarrow before being fed on the ground. The daily feeding regime for each horse was two flakes (~1.59 kg, as-is) of Ontario grown first-cut mixed timothy-alfalfa hay three times a day (0600h, 1200h, 1700h), along with a grain and concentrate mixture. This mixture was tailored for each individual horse and kept consistent throughout the study.

**Experimental design**

The experiment was designed as a 3 × 3 incomplete Latin square design, in which animals were split into three cohorts to allow for video-taping. Each cohort completed the feeding trial over 24 days in two phases; first a conditioning phase and then the preference testing phase. The conditioning phase consisted of each horse being offered 1.59kg of treatment hay from a hay bag in replacement of their normal hay at two of their normal feeding times, 1200h (lunch) and 1700h (dinner), for five consecutive days. This was repeated until horses had
been offered all three hay treatments, such that the conditioning phase lasted for 15 consecutive days. The order of treatments offered was randomized by cohort. Next, the horses completed the preference testing phase over nine days. Horses were simultaneously offered two hay treatments at lunch and dinner for three consecutive days, then offered a different pairing of hay treatments for the next three days, and the final possible pairing of treatments for the last three days. The order of treatment combinations offered was randomized by cohort.

**Treatments**

For every preference test, a canvas hay bag was weighed before filling with hay and weight was recorded. When offering dry hay, hay was left as-is and placed directly in canvas hay bag by hand and 1.59 kg of dry hay (1.38kg DM) was weighed out for each horse. Steamed hay was prepared by placing a fully strung small square hay bale (~17.5kg as-is) in a commercial hay steamer (HG-2000; Haygain Ltd, Lambourn, Berkshire, UK) and steamed according to manufacturer’s instructions (steamer run for approximately one hour, such that the temperature gauge fell between 74°C and 85°C for at least 10 minutes). Upon completion of the steamer cycle, the bale was removed, placed on a clean, dry tarp, strings were cut, and flakes were spread across the tarp to promote more rapid cooling. Flakes were flipped over after five minutes to further enable cooling and left for another five minutes. Steamed hay was then placed in canvas hay bags and 1.59kg of hay (1.37kg DM) was weighed out for each horse as steaming did not change the moisture content of the hay. The quantity of soaked hay that was offered to each horse had to be altered from 1.59kg to 0.91kg of dry hay to minimize wastage, because it was determined within the first couple of days of the conditioning phase (results not published) that even after one hour, no horses had finished all of their soaked hay. For the rest of the conditioning phase and for all of the preference testing phase, soaked hay was prepared by
weighing out 0.91kg of dry hay (0.79kg DM) in nylon hay nets after accounting for the weight of the hay net, then placing all of the filled hay nets in one clean plastic bin, and filling the bin with room temperature water from a tap inside the barn. Hay nets were left to soak for 30 minutes, then hung to drip dry for another 30 minutes and then transferred from each hay net to a hay bag and weighed again to account for how much water had been absorbed before being fed.

Three “all over” grab samples were collected from steamed hay and three from soaked hay during feeding trials for nutrient analyses to represent “daily protocol” treatments in the protocol comparison.

Conditioning protocol

In preparation for the preference testing, two hooks were screwed into each stall on the left wall, relative to the research when looking into the stall. The hooks were placed approximately 1m apart from each other, such that two hay bags could be hung side by side, but with enough space between them such that the horse would need to make an obvious movement to eat from one or the other. Hooks were placed so that when hay bags were hung the feeding hole of the hay bag was approximately 1.5m from the ground, which was roughly level with the horse’s mouth when in a standing position. During the conditioning phase, horses were offered one hay bag with the designated treatment hay at each feeding period, so the hook that the bag was placed on was altered daily to acclimatize the horse to eating from both locations. Since eating from the hay bag was a new experience to all of the horses, the bags offered at the lunch feeding were left up from 1200 to 1530 h, when the researchers returned to prepare for the dinner feeding, to allow horses more time to acclimatize to eating from the hay bags. At the dinner feeding, hay bags were only left up for one hour because of safety concerns over leaving them up overnight. After taking hay bags down, they were immediately weighed, and any hay left in the
bag was then emptied onto the floor of the horse’s stall, in the same manner they had been fed prior to the study. The empty bags were then weighed again so that the quantity of hay consumed could then be determined.

**Preference test protocol**

Treatment hay for the preference test was prepared in the same manner as for the conditioning period and was offered from the same canvas hay bags. For the preference testing, two hay bags were prepared and hung up for each horse at each feeding, so that two different treatments were offered side by side. A video recording device (AKASO 4K ultra HD model EK7000 action cameras, Panasonic HC-X900M 16MP camcorder, and Sony Handycam HDR-CX405 9.2MP) was set up at the top of the front of each stall to record horses while eating. When hay bags were ready, one researcher would enter the stall and hold the horse by the halter off to the right side of the stall with the horse facing towards the front of the stall. Once the horse was secured, the second researcher would hang the hay bags. A random number generator was used to ensure that all treatment combinations were done twice, with treatments provided on both the left and right hooks to account for side preference. Once the bags were hung, the second researcher exited the stall and started the video recorder, then the first researcher would let go of the horse and exit the stall. The second researcher would record what time the video recording started and when the horse had been released, as well as which hay bag the horse approached first and ate from first. This exact procedure was duplicated for each horse. After each horse had been given 30 min to eat, the first researcher would enter the stall and secure the horse to the right of the stall while the second researcher stopped the video recording, took down the hay bags, and took them out of the stall. Hay bags were weighed in the same manner as the conditioning period to determine total hay intake, which was used to calculate dry matter intake.
When calculating dry matter intake, the average dry matter content of dry and steamed hay was used for those respective calculations. For soaked hay, the moisture content of the hay before soaking was assumed to be the average moisture content of dry hay, while the added water content of soaked hay was determined from the difference in weight of the hay before and after soaking.

**Coding of video-recordings**

An unbiased person, who was not involved in data collection or the study in general, was assigned the task of recording all assigned video names and relevant data, and renaming the videos using a random word generator so that two researchers from the study could code the videos and be blinded against which hay treatments were being offered and which horse was in the video. The videos were coded using Behavioural Observation Research Software (BORIS, version 6.3.1., University of Torino, Italy). BORIS was also used to calculate the Cohen’s kappa statistic, to assess inter-rater reliability after both researchers had separately coded the same video. There was a total of 234 videos, with each video encompassing the 30-min feeding period. Inter-rater reliability was calculated from 46 videos (20% of total videos) and the remaining 188 videos (80% of total) were randomly split between the two researchers. The researchers first coded 10 of the same videos (4% of total) to ensure their Cohen’s kappa statistic was over 80%. Then their patterns were to code 15 of their own set of videos (6% of total), then code two more of the shared videos (1% of total) to ensure their Cohen’s kappa statistic was still over 80%. This pattern was repeated until all of the videos were coded. The ethogram that was created to code the behavioural events of the videos was based on which side the relevant hay bag was on from the horse’s point of view, and included total time spent investigating each side and total time spent eating from each side.
5.3.3. Glycemic response tests

Horses

Nine healthy, privately-owned, Standardbred racehorses (463 ± 45 kg, mean ± SD; 5 geldings, 4 mares; 1.5 – 9 years of age) participating in the feeding experience trials were also selected for the glycemic response tests and allocated to treatment rotation blocked by sex and age. All horses used in the glycemic response tests had not competitively raced in the past 72 hours prior to each glycemic test. Horses had all undergone their regular training the day before, with the exception of one horse which was on stall rest for the duration of the study. Horses were housed individually in 3 × 3 m box stalls with wood shavings for the duration of the experimental period.

Experimental design

The nine horses were randomly split into three groups and the experiment was conducted as a 3 × 3 Latin square design, with three treatments (dry, steamed, and soaked hay). Due to conflicts with racing schedules and equipment issues, three horses were unavailable to participate on the first trial date and completed the study on a delayed schedule starting on the second trial date, one week later. Glycemic response testing was conducted once a week for three consecutive weeks, always on the same day each week when horses did not undergo their regular morning training.

Treatments
Each horse received 0.5% BW of the dry weight equivalent of treatment hay at the beginning of each trial, following a 10-hour fast. This was accomplished by weighing out dry hay for each horse, then subjecting it to the appropriate treatment the night before the trial. For dry, hay was left as is. For steamed, hay was steamed the night before the trial in nylon hay nets in a commercial hay steamer (HG-2000; Haygain Ltd, Lambourn, Berkshire, UK) according to manufacturer’s instructions (steamer run for approximately one hour, such that the temperature gauge fell between 74°C and 85°C for at least 10 minutes). Upon completion of the steamer cycle, the steamer chest was kept closed to best retain moisture until the next morning. For soaked, hay nets were placed in a clean plastic bin filled with room temperature water. Nets were left to soak overnight for 10 hours, then hung to drip dry for 30 minutes in the morning before being fed.

Three “all over” grab samples were collected from steamed hay and three from soaked hay during glycemic response trials for nutrient analyses to represent “overnight protocol” treatments in the protocol comparison.

**Blood collection and glucose analyses**

The night before each trial, all participating horses received their last meal (hay and grain) at 1700 h. Researchers arrived at 0415 h the next morning to complete trial setup. Soaked hay was hung up to drain, horses were haltered, topical anesthetic (EMLA® Cream (2.5% lidocaine & 2.5% prilocaine), Astra Pharmaceuticals, L.P. Wayne, PA) was applied to the sampling area on the neck, and baseline blood samples were collected. Horses were then fed treatment hay at 0500 h (time 0) and blood samples were collected every 30 minutes for the next 6 hours. All blood samples were taken from the jugular vein, at least 10 minutes after application of topical anesthetic, from alternating sides every hour. For each sampling, approximately 2mL
of blood was collected in lithium heparin vacutainers (Becton, Dickinson, and Company, Franklin Lakes NJ, USA) using 21G × 1.5” needles, and samples were gently inverted several times as directed. Whole blood glucose levels were determined immediately using a handheld glucose monitoring system (AlphaTRAK; Abbott Laboratories, Abbott Park IL, USA) validated for use in horses (Hackett and Mccue, 2010). After the final samples were collected at 1100 h, horses were fed their afternoon meal (hay and grain) and resumed their regular daily schedule.

5.3.4. Statistical analyses

All nutrient data were analyzed using the generalized linear mixed model procedure of a commercial software (SAS version 9.4; SAS Institute, Cary NC, USA). To assess effects of treatment alone on nutrient density of the hay, regardless of position sampled from, treatment (dry, steamed, soaked) was a fixed effect and bale was used as a random effect to account for samples taken from different bales of hay. This analysis was then repeated separately for each treatment, with position (top, middle, bottom) as the fixed effect and bale as the random effect. Finally, effects of differing treatment protocols were assessed for each experimental treatment (steamed, soaked), with protocol (daily, overnight) declared as fixed effects and bale as the random effect. All results were expressed as least squares means ± standard error of the mean (s.e.m.) and differences were considered significant at \( P \leq 0.05 \).

Behaviour data were analyzed using the generalized mixed model procedure of SAS and results expressed as least squares means ± s.e.m. In all cases, differences were considered significant at \( P \leq 0.05 \). Data from each trial were analyzed separately based on combinations of treatments that were presented. The variables “day offered” (i.e. first, second, or third day that horse was presented with that particular combination) and “meal” (lunch or dinner) were combined into one variable “daymeal”. For each combination, dry matter consumed, time spent
eating, and time spent investigating were analyzed using the generalized mixed model procedure with treatment, side, and daymeal as the fixed effects, trial (1\textsuperscript{st} – 6\textsuperscript{th} for each combination; i.e. dinner on the second day of that combination would be the 4\textsuperscript{th} trial) as the random effect, and horse as the subject.

All glycemic response data were analyzed using the mixed model procedure of SAS and results expressed as least squares means ± s.e.m. In all cases, differences were considered significant at $P \leq 0.05$. Blood glucose variables were analyzed using repeated measures in a mixed model with treatment, time, and the interaction of treatment and time as the fixed effects, and horse nested in treatment as the random effect. The subject was horse. The variance-covariance matrix used was a Toeplitz matrix and was selected based on the lowest value for Akaike information criterion (AIC). The area under the curve (AUC), time to peak, and peak value data was analyzed using a mixed model with treatment as a fixed effect and horse as a random effect. AUC was calculated using the trapezoidal method.

5.4. Results

5.4.1. Hay nutrient analyses

Effect of treatment on nutrient composition

Table 5.1. shows the nutrient content of each treatment hay expressed as a percent of total contents on a dry matter basis (DMB). The moisture content of soaked hay was four times that of dry ($P<0.0001$), and there was no difference between dry and steamed hay ($P>0.05$). Crude protein content was greater in soaked hay compared to dry and steamed hay ($P=0.0239$). Soluble protein content was lesser in soaked hay compared to steamed hay ($P=0.0082$) but was not different compared to dry hay ($P>0.05$). Soaking resulted in a greater proportion of ADF in
comparison to dry and steamed hay ($P=0.0024$) but had no effect on aNDF content ($P>0.05$). Lignin content was greatest in soaked hay, intermediate in dry hay, and least in steamed hay ($P<0.0001$). Both steaming and soaking led to greater proportions of fat compared to dry hay, and fat was greater in soaked hay than steamed hay ($P=0.0018$). Soaking resulted in lesser proportions of WSC, ESC, NSC, and starch by 32%, 25%, 29%, and 17% respectively compared to dry hay ($P<0.0001$, $P<0.0001$, $P<0.0001$, $P=0.0045$, respectively), while there were no differences between steamed hay and dry hay ($P>0.05$). Soaking, however, resulted in 32% greater calcium content ($P=0.0022$) and no difference in phosphorus content ($P>0.05$), resulting in a greater calcium to phosphorus ratio ($P<0.0001$). Steaming had no effect on these nutrients in contrast to dry hay ($P>0.05$). Potassium was different among all three hay treatments, with the least in soaked hay and greatest in steamed hay ($P<0.0001$). There was also greater sodium content in soaked hay compared to dry and steamed hay ($P=0.0164$). Copper was greater in soaked hay compared to steamed hay ($P=0.0414$), but neither was different from the dry hay ($P>0.05$). Magnesium, iron, manganese, and zinc were not different among treatments ($P>0.05$).
Table 5.1. Forage nutritive concentrations of timothy-alfalfa hay when dry, soaked for 30 min, and steamed for 60 min; reported on a DMB (mean ± s.e.m.).

<table>
<thead>
<tr>
<th></th>
<th>Soaked</th>
<th>Dry</th>
<th>Steamed</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry Matter, % as-is</td>
<td>29.9 ± 0.9b</td>
<td>86.7 ± 1.0a</td>
<td>86.0 ± 0.4a</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Moisture %, as-is</td>
<td>70.1 ± 0.9a</td>
<td>13.3 ± 1.0b</td>
<td>14.1 ± 0.4b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Crude Protein, % DM</td>
<td>9.4 ± 0.4a</td>
<td>8.3 ± 0.4b</td>
<td>8.4 ± 0.3b</td>
<td>0.0239</td>
</tr>
<tr>
<td>Soluble Protein, % DM</td>
<td>2.6 ± 0.3b</td>
<td>3.2 ± 0.3ab</td>
<td>3.4 ± 0.2a</td>
<td>0.0082</td>
</tr>
<tr>
<td>ADF, % DM</td>
<td>46.0 ± 0.8a</td>
<td>43.2 ± 0.9b</td>
<td>43.0 ± 0.6b</td>
<td>0.0024</td>
</tr>
<tr>
<td>aNDF, % DM</td>
<td>66.8 ± 1.6</td>
<td>65.7 ± 1.7</td>
<td>65.5 ± 1.5</td>
<td>0.2712</td>
</tr>
<tr>
<td>Lignin, % DM</td>
<td>11.2 ± 1.0a</td>
<td>9.7 ± 1.0b</td>
<td>8.8 ± 1.0c</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Fat, % DM</td>
<td>1.6 ± 0.1a</td>
<td>1.2 ± 0.2c</td>
<td>1.4 ± 0.2b</td>
<td>0.0018</td>
</tr>
<tr>
<td>ESC, % DM</td>
<td>4.9 ± 0.6b</td>
<td>6.5 ± 0.6a</td>
<td>6.9 ± 0.5a</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>WSC, % DM</td>
<td>6.7 ± 0.5b</td>
<td>9.8 ± 0.6a</td>
<td>9.9 ± 0.2a</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>NSC, % DM</td>
<td>8.8 ± 0.6b</td>
<td>12.4 ± 0.7a</td>
<td>12.4 ± 0.3a</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Starch, % DM</td>
<td>2.1 ± 0.2b</td>
<td>2.5 ± 0.2a</td>
<td>2.6 ± 0.2a</td>
<td>0.0045</td>
</tr>
<tr>
<td>Ash, % DM</td>
<td>4.9 ± 0.7ab</td>
<td>4.7 ± 0.7b</td>
<td>5.5 ± 0.6a</td>
<td>0.0657</td>
</tr>
<tr>
<td>Calcium, % DM</td>
<td>0.6 ± 0.0a</td>
<td>0.5 ± 0.0b</td>
<td>0.5 ± 0.0b</td>
<td>0.0022</td>
</tr>
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</tr>
<tr>
<td>Phosphorus, % DM</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.6411</td>
</tr>
<tr>
<td>Ca:P, DM</td>
<td>4.0 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Potassium, % DM</td>
<td>0.8 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.4 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Sodium, % DM</td>
<td>0.1 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0164</td>
</tr>
<tr>
<td>Magnesium, % DM</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.5507</td>
</tr>
<tr>
<td>Copper, ppm DM</td>
<td>5.7 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.9 ± 0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.9 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0414</td>
</tr>
<tr>
<td>Iron, ppm DM</td>
<td>118.6 ± 76.9</td>
<td>77.9 ± 84.8</td>
<td>187.9 ± 52.2</td>
<td>0.4022</td>
</tr>
<tr>
<td>Manganese, ppm DM</td>
<td>53.5 ± 24.0</td>
<td>47.5 ± 24.2</td>
<td>52.1 ± 23.7</td>
<td>0.7260</td>
</tr>
<tr>
<td>Zinc, ppm DM</td>
<td>21.2 ± 1.2</td>
<td>18.3 ± 1.3</td>
<td>20.1 ± 0.6</td>
<td>0.2874</td>
</tr>
<tr>
<td>Zn:Cu, DM</td>
<td>3.8 ± 0.3</td>
<td>3.7 ± 0.3</td>
<td>4.2 ± 0.1</td>
<td>0.2578</td>
</tr>
</tbody>
</table>

Abbreviations: s.e.m., standard error of the mean; DM(B), dry matter (basis); ADF, acid detergent fibre; aNDF, a-neutral detergent fibre; ESC, ethanol-soluble carbohydrates; WSC, water-soluble carbohydrates; NSC non-structural carbohydrates; Ca:P, calcium to phosphorus ratio; Zn:Cu, zinc to copper ratio

<sup>a, b, c</sup> Least square means within a row not sharing a common superscript letter differ (\( P \leq 0.05 \)), p-value refers to the analysis of variance of the effects of treatment.
Effect of position on treatment

Greater moisture was found in the bottom section of the soaked hay compared to the top position ($P=0.0177$). No other significant position effects were observed in the soaked hay and none were observed in the steam treated hay ($P>0.05$).

Effect of treatment protocol on hay

Differences were found between the daily and overnight protocols for both steamed and soaked hay. Hay that was soaked for 10 hours had 22% less crude protein than hay soaked for 30 minutes ($P=0.0066$). Interestingly, WSC and NSC were greater in overnight 10-hour-soaked hay than 30-min-soaked hay, by 47% ($P=0.0284$) and 39% ($P=0.0222$), respectively. Potassium and sodium were lesser for the overnight 10-hour-soak compared to the 30-min-soak, by 41% ($P=0.0496$) and 81% ($P=0.0213$), respectively. No other differences between soaking times were observed. Hay left in the steamer chest overnight for 10 hours had greater moisture ($P=0.0443$) and WSC ($P=0.0252$) and less soluble protein ($P=0.0015$) content than hay removed immediately from the steamer and allowed to cool in the open. There were no other differences found between the different steaming protocols ($P>0.05$).

Water mineral analyses

Table 5.2. displays the mineral content of the water used to soak the hay, alongside the mineral content of the soaked hay as-is, and the calculated contribution of minerals from soaking water to the hay. These findings demonstrate that negligible amounts of minerals (<5% of contents in hay) may have been introduced to soaked hay from water.
Table 5.2. Mineral contents of hay soaking-water and soaked hay on an as-is basis (mean±s.e.m.), and calculated contribution of water mineral content to soaked hay assuming that the moisture content of soaked hay was entirely tap water (70%).

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Water contents</th>
<th>Soaked hay contents</th>
<th>% of minerals in hay from water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium, % as-is</td>
<td>84.85E-04 ± 1.78E-04</td>
<td>0.18±0.007</td>
<td>3.31</td>
</tr>
<tr>
<td>Phosphorus, % as-is</td>
<td>&lt;0.1E-04</td>
<td>0.05±0.007</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Potassium, % as-is</td>
<td>3.77E-04 ± 0.72E-04</td>
<td>0.25±0.017</td>
<td>0.11</td>
</tr>
<tr>
<td>Sodium, % as-is</td>
<td>9.18E-04 ± 0.09E-04</td>
<td>0.02±0.001</td>
<td>3.22</td>
</tr>
<tr>
<td>Magnesium, % as-is</td>
<td>33.27E-04 ± 0.36E-04</td>
<td>0.05±0.007</td>
<td>4.67</td>
</tr>
<tr>
<td>Copper, ppm as-is</td>
<td>&lt;0.1</td>
<td>1.69±0.08</td>
<td>&lt;4.15</td>
</tr>
<tr>
<td>Iron, ppm as-is</td>
<td>0.43±0.04</td>
<td>33.52±6.17</td>
<td>0.90</td>
</tr>
<tr>
<td>Manganese, ppm as-is</td>
<td>0.13±0.01</td>
<td>15.08±4.84</td>
<td>0.60</td>
</tr>
<tr>
<td>Zinc, ppm as-is</td>
<td>0.05±0.01</td>
<td>6.26±0.31</td>
<td>0.56</td>
</tr>
</tbody>
</table>

5.4.2. Feeding experience study

Preference tests

Table 5.3. shows the dry matter intake, total time spent eating, and total time spent investigating for each hay treatment in the 30-min two-choice preference tests. One horse refused to eat from the hay bags and was removed from the study; therefore, 13 horses completed the preference testing component of the study. Soaked hay was eaten less when compared to both dry (P=0.0086) and steamed (P=0.0096) hay on a dry matter basis, and there was no difference between the amount of hay eaten when dry and steamed were offered together (P>0.05). There
were no differences between any combinations of treatments for time spent investigating hays. Less time was spent eating soaked hay when offered with dry ($P=0.0026$), and there was no difference between time spent eating when steamed and dry were presented together, or when steamed and soaked were presented together ($P>0.05$).

Table 5.3. Average amount of hay consumed, time spent eating, and time spent investigating (mean ± s.e.m.) when two treatment hays were offered simultaneously for 30 minutes to horses (n=13).

<table>
<thead>
<tr>
<th></th>
<th>Dry</th>
<th>Steamed</th>
<th>Soaked</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dry Matter Intake</strong></td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.068</td>
<td></td>
</tr>
<tr>
<td><strong>Intake (kg)</strong></td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.0093</td>
<td></td>
</tr>
<tr>
<td><strong>Time Spent Eating</strong></td>
<td>824.4 ± 88.6</td>
<td>544.2 ± 88.6</td>
<td>0.0018</td>
<td></td>
</tr>
<tr>
<td><strong>(seconds)</strong></td>
<td>860.7 ± 225.7</td>
<td>667.9 ± 225.7</td>
<td>0.0489</td>
<td></td>
</tr>
<tr>
<td><strong>Time Spent Investigating</strong></td>
<td>23.5 ± 9.2</td>
<td>15.6 ± 9.2</td>
<td>0.2879</td>
<td></td>
</tr>
<tr>
<td><strong>(seconds)</strong></td>
<td>27.3 ± 13.5</td>
<td>34.1 ± 13.5</td>
<td>0.1688</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.3 ± 5.4</td>
<td>17.1 ± 5.4</td>
<td>0.3999</td>
<td></td>
</tr>
</tbody>
</table>

*a, b.* Least square means within a row not sharing a common superscript letter differ ($P \leq 0.05$), $p$-value refers to the analysis of variance of the effects of treatment.
5.4.3. Glycemic response tests

All nine horses completed a 6-hour blood glucose response trial for each hay treatment. There was a change, due to hay consumption, in blood glucose over time within all treatments ($P<0.0001$) as expected. Average blood glucose for horses fed dry hay was 5.3±0.2mmol/L, soaked hay was 5.2±0.2mmol/L, and steamed hay was 5.4±0.2mmol/L. There were no differences in blood glucose among treatments or among treatments over time. Average total AUC for horses fed dry hay was 1894.6±54.7mmol/L·360min, fed soaked hay was 1876.6±54.7mmol/L·360min, and fed steamed hay was 1852.3±54.7mmol/L·360min. Average time to peak and peak glucose value for horses fed dry hay were 187±40min and 6.2±0.2mmol/L respectively, soaked hay were 213±40min and 5.8±0.2mmol/L respectively, and steamed hay were 187±40min and 5.6±0.2mmol/L respectively. There was no difference between AUC, peak time, or peak glucose value among treatments ($P>0.05$).

5.5. Discussion

This was the first study to look at the effects of steaming and soaking on nutrient loss in Ontario-grown timothy-alfalfa hay, whether these changes in nutrient content occur evenly throughout the hay, a comparison of the glycemic response of performance horses when consuming these hays, and their preferences for them. Overall, in contrast to dry hay, soaking resulted in substantial nutrient losses that could be detrimental to a horse with high energy demands like racehorses. The commercial steamer evenly treated hay and conserved nutrients. In addition, performance horses displayed tight control over blood glucose levels, regardless of NSC/WSC levels of hay consumed, and least preferred soaked hay when either dry or steamed was available. Overall, this suggests that when comparing dust-reducing treatments (Blackman
and Moore-Colyer, 1998; Moore-Colyer and Fillery, 2012), steaming would be better for maximizing nutrient intake from hay, in contrast to soaking.

Prior to this study, Moore-Colyer & Payne (2012) were the first researchers to investigate horse preferences for dry, steamed, and soaked hay, using timothy-fescue hay and six Polo ponies by simultaneously offering all three treatments at once, and concluded that the ponies consumed more steamed hay than dry and more dry hay than soaked. Differences between their experimental design and ours may have led to slightly differing results, including their smaller sample size, offering three treatments at once rather than two, their previous diet consisting of haylage rather than hay, and unknown conditioning parameters. On the other hand, Pagan et al (2013) took a different approach and assessed intake of each type of treatment hay when offered separately to three Thoroughbreds and reported results similar to ours; that steamed and dry intake were greater than that of soaked hay. The results of these studies, in combination with ours, suggest that performance horses prefer steamed and dry hay over soaked hay. Together, this suggests that horses with very high dietary energy requirements would be best served by providing steamed hay to help maximize dry matter intake, while reducing dust (Blackman and Moore-Colyer, 1998; Moore-Colyer and Fillery, 2012).

We expected to see some variation in nutrient losses compared to those seen in UK and US grown hays due to the different characteristics of the hays themselves. Since grass hays typically have less protein (Longland et al., 2011), while legumes have greater protein (Martinson et al., 2012a), the mid-range level of crude protein seen in the current study was expected for a hay that is a mix of these two hay types. In agreement with this, the soluble protein level in the mixed timothy-alfalfa hay was greater than those seen in grass hays by Longland et al (2009). Having more soluble protein to lose may explain why there was a greater
loss of soluble protein on a percentage basis in the current study than in the grass hays studied by Longland et al (2009). Interestingly, soluble protein content was greater in the hay that was steamed, and this reflects the improved protein digestibilities reported by other researchers who have investigated the effects of thermal processing on plant-based food sources (Broderick et al., 1993; Rehman and Shah, 2005). However, overly processing hay has the potential for protein degradation, and this is likely why soluble protein was less in the present study when hay was steamed by the overnight protocol and the steamer chest was kept closed, exposing hay to the heat for longer. Future studies should investigate the optimal steaming time and temperature to reach maximum protein availability.

Researchers have reliably seen decreases in WSC content in hay soaked for longer than ten minutes (Warr and Petch, 1992; Blackman and Moore-Colyer, 1998; Longland et al., 2009; Longland et al., 2011; Martinson et al., 2012b; Longland et al., 2014; Mack et al., 2014; Moore-Colyer et al., 2014; Hansen et al., 2016; Müller et al., 2016); this is consistent with the 32% decrease of the WSC levels in timothy-alfalfa in the current study. American first-cut orchardgrass hay also had a 32% loss in WSC content after a 30 minute soak in cool water, and a 27% loss in first-cut alfalfa hay (Martinson et al., 2012b). Warr & Petch (1992) examined changes of WSC content in UK hay after soaking and saw a 31% decrease after 30 minutes. When both of these research teams soaked hay for longer (eight hours) they observed double the loss in WSC content (Warr and Petch, 1992; Martinson et al., 2012b), but this was not the case in the current study. Water temperature may have been an important factor influencing this group of nutrients, since temperature remained constant in the above-mentioned studies. It could not be controlled in the current study because we used tap water from the barn that was not temperature-regulated, and hay was soaked overnight in the unheated barn during late fall in
southern Ontario, Canada. We believe that colder temperatures overnight resulted in colder soaking water temperatures, compared to hay soaked during the day. Soaking hay for 60 minutes in cold water extracts the same amount of soluble carbohydrates as 30 minutes in warm water (Watts and Sirois, 2003), therefore the removal of WSC and NSC would not have been as efficient in the cooler water overnight.

Research on nutrient content changes in steamed hay have shown conflicting results concerning WSC content, ranging from a 7% increase to an 18% decrease and with no clear correlation to initial WSC content or steaming protocol. In the current study, WSC levels in steamed hay were 1.5% greater than in dry, but this difference was not significant. When steaming protocols were compared, moisture and WSC content were higher in the overnight protocol hay compared to the daily protocol hay. This likely occurred because the steamer chest was kept closed overnight and instead of evaporating, the steam condensed on the lid of the steamer chest and eventually dripped back onto the hay inside the chest, returning water back onto the hay, which consisted of moisture and WSC.

Mammals rely heavily on glucose, stored as glycogen in the liver and muscle tissue, and as an energy source during exercise (Harris, 1997). Lacombe et al (2004) found that feeding a higher glycemic index diet post-exercise resulted in a higher muscle glycogen concentration, as well as a faster rate of glycogen synthesis that is beneficial to recovery and replenishment of energy stores. In the current study the level of NSCs in soaked hay were considered moderately low (less than 10%), whereas steaming conserved these nutrients (greater than 12%); thus, steamed hay may be beneficial for a horse performing high intensity exercise. Future work should consider evaluating whether differences in WSC influence muscle glycogen content.
While starch, NSC, WSC, and ESC were not different between steamed and dry hay, all of these nutrients were less in soaked hay, and it was hypothesized that hay with lower carbohydrate contents would elicit a lower glycemic response in horses, as was reported by Cottrell et al (2005) in their study using four- to five-month old fillies. This, however, was not the case in the current study, which used exercise-conditioned Standardbred racehorses. Our results agree with those of Collins (2015), who investigated glycemic response in mature horses (13-21 years of age) and also observed no difference in glycemic response despite lower NSC content in soaked hay. Borgia et al (2009) looked at glycemic and insulninemic response in both healthy and polysaccharide storage myopathy (PSSM) horses after consuming hays with either low or high NSC content and reported higher glucose and insulin responses after consuming high NCS hay; however, in healthy horses consuming high NSC hay a heightened response was only detected in insulin, not glucose and supports the current observations. This indicates that insulin may be a more sensitive biological marker, and if it had been measured in addition to glucose, might have revealed the predicted response changes. This may have been especially true for our particular study participants, as exercise-conditioned horses, like other athletes, are known to have greater insulin-sensitivity (Pratt et al., 2006) in contrast to sedentary individuals. This would explain how they tightly regulated their blood glucose levels despite differing carbohydrate intakes. Future research would more greatly benefit horse owners by investigating the glycemic responses of insulin-resistant horses after consuming soaked, steamed, and dry hay.

A variety of mineral content changes were noted in both soaked and steamed hay, many of which may have a notable impact on performance horses. Among these, potassium and sodium are regarded as some of the most important minerals measured in this study, due to their roles as electrolytes. Potassium levels of timothy-alfalfa hay in this study decreased by 41% after
a 30-minute soak when compared to the dry hay, which is very similar to the 40% decrease seen in UK meadow hay after a 30-minute soak (Blackman and Moore-Colyer, 1998). This was not surprising since potassium in hay has been shown to be more soluble than carbohydrates (Watts and Sirois, 2003), and in the current study a greater proportion of potassium was lost than WSC. While potassium was decreased by soaking hay, the proportion of sodium nearly doubled, though it still only made up 0.07% of the dry matter, whereas potassium decreased from 1.43% to 0.84% of dry matter. In addition, the lower levels of potassium and sodium in the overnight (10 hours) soaked hay compared to the 30-minute soaked hay are consistent with past studies where a decrease of 23% and 16%, respectively, were reported when comparing a one hour immersion to an eight hour immersion (Hansen et al., 2016), and suggests that minerals in timothy-alfalfa hay are more susceptible to time-dependent leaching than carbohydrates.

Changes in the levels of potassium and sodium in the diet need to be carefully monitored when feeding the equine athlete, as these minerals play a vital role in muscle fibre contraction and exercise recovery (Gottlieb-Vedi et al., 1996). Athletic horses are at a particular disadvantage if their diet is not sufficient in these nutrients, since horse sweat is hypertonic, and this can be exacerbated when horses are worked harder or longer since the extent of total body electrolyte loss is dependent on the intensity and duration of the activity (Ecker and Lindinger, 1995).

Interestingly, potassium levels were increased in steamed hay and there was no change seen in sodium levels. As has been demonstrated in other studies, it appears that heat treatment increases the availability of potassium in hay (Blackman and Moore-Colyer, 1998; Moore-Colyer et al., 2016), which would be beneficial for an athletic horse.

Another mineral of interest was calcium, which increased by 32% in soaked hay, likely a reflection of the loss of other nutrients and subsequent proportional increase of calcium.
Consequently, the Ca:P ratio increased from 2.85:1 to 4.03:1, since phosphorus levels were the same in dry and soaked hay, indicating a small loss of phosphorus from soaking. This drastic change in calcium content was unexpected since Martinson et al (2012b) were the only other researchers to report an increase in calcium content after soaking, and the largest increase was only 6% in second-cut orchardgrass hay after both a 15- and 30-minute soak in cool water. Prior to this study, changes measured in calcium content have ranged from increases of 3%, to decreases of 9% (Moore-Colyer, 1996; Blackman and Moore-Colyer, 1998; Martinson et al., 2012a), while Mack et al (Mack et al, 2014) reported a loss of 22% in UK hay. Steaming on the other hand, did not affect calcium or phosphorus and the Ca:P ratio was maintained and similar to dry hay. The changes in mineral content seen in soaked hay will be of concern to performance horse owners and future research should assess the metabolic implications of micronutrient loss in soaked versus steamed or dry hays.

5.5.1. Conclusions

Soaking hay reduced NSC (including WSC, ESC, and starch) content of Canadian first-cut mixed timothy-alfalfa hay, as anticipated. Equine athletes have very high energy and nutrient demands and steaming hay was an effective method to conserve these nutrients to maximize nutrient intake. These horses also preferred steamed or dry hay over soaked hay, further supporting a recommendation that steaming is a superior method for treating hay for performance horses.
6. Use of Alimet® (methionine hydroxy analogue) to reduce bacterial contamination in raw meat for pet diets

6.1. Abstract

While the raw pet food market continues to grow, the risk of bacterial contamination in these types of diets is the major concern, with *Salmonella enterica* and *Listeria monocytogenes* being the most frequent bacteria identified in raw pet product recalls. Since organic acids are commonly applied to raw carcasses in the meat industry to combat bacterial contamination, there is the potential for a type of organic acid already included in pet food to have an additional use for this purpose. DL-methionine is included in some commercial feline kibble and canned diets to improve protein quality; however, an alternative to this is a liquid methionine supplement, methionine hydroxy analogue (Alimet®) which is also an organic acid. Alimet® has previously demonstrated similar efficacy to formic acid against pathogens in a liquid environment and may be a good candidate to inhibit *S. enterica* and *L. monocytogenes* in a raw ground meat environment. Initially the minimum inhibitory concentration (MIC) of Alimet® against these pathogens under laboratory growth conditions was determined by measuring growth of pathogens over 48 hours when exposed to 10 levels of Alimet® (0.1 – 1.0%) mixed with tryptic soy broth. Alimet® included as ≥0.5% was bactericidal to *S. enterica* and *L. monocytogenes* (P<0.05). Next, 5 levels of Alimet® (0.50% - 1.25%) were included in raw ground meat mixtures inoculated with cocktails of *S. enterica* or *L. monocytogenes*, and contamination levels were determined at four timepoints: immediately, after refrigerated storage (4°C) at 24h, 48h, and 72h after removal from freezer (24h at -20°C). Alimet® included as 1.25% of the meat mixture reduced *S. enterica* and *L. monocytogenes* compared to the control (P<0.05); however, it did not result in total kill of either of these pathogens. Future research investigating the efficacy
of higher concentrations of Alimet® or Alimet® in conjunction with other hazard control methods such as longer freeze time or lower temperature is warranted.

6.2. Introduction

The raw meat-based diet (RMBD) market for dogs and cats continues to increase rapidly (Wall, 2018), but these diets are discouraged by veterinarians due to the risk of bacterial contamination (CVMA, 2018). In 2018, there were 35 recalls of pet food and treats by the United States Food and Drug Administration (FDA), 22 of which were for raw products. All raw recalls were for bacterial contamination concerns, while none of the heat-treated product recalls were related to bacteria. Of the 22 raw product recalls, 21 involved Salmonella spp., Listeria monocytogenes, or both (FDA, 2018). Although there is a zero-tolerance policy for bacterial contamination in pet food in the United States (FDA, 2016), this is not the case in Canada. As such, the only recalls of raw pet foods in Canada have been voluntary recalls initiated by the manufacturer themselves or recalls for imported pet foods which have been recalled by the FDA (PHAC, 2018). Researchers have identified pathogens in Canadian raw pet food including Salmonella spp., E. coli spp., Clostridium perfringens, Clostridium difficile, and Staphylococcus aureus (Weese et al., 2005; Finley et al., 2008; Lefebvre et al., 2008; Leonard et al., 2011), and Canadian pet owners should be concerned by this.

In the United States, some manufacturers of raw pet food utilize high-pressure processing (HPP) in the production of their pet food (Stewart, 2014; Bravo, 2015; Stella & Chewy’s, 2017; Northwest Naturals, 2018; Instinct, 2019; Primal Pet Foods, 2019; Tucker’s, 2019). HPP is a technology requiring expensive specialized equipment that can work without heat to reduce bacterial contamination using pressure (Gola et al., 2000). The effectiveness of HPP was investigated in raw beef-based diets inoculated with 7 logs of non-pathogenic E. coli strains that
have been validated as substitutes for pathogenic *E. coli* and *Salmonella* spp. (Hasty, 2017). Samples tested 24h after HPP demonstrated a 4.9 log reduction of *E. coli*, indicating HPP is a moderately reliable technology to reduce pathogens in raw meat; however, HPP alone may not result in total kill of pathogens. In addition, as not all raw pet food manufacturers have access to this technology, more accessible options are worth investigating.

One such option is the application of organic acids, which are commonly used in the meat industry post-slaughter to reduce carcass contamination (Theron and Lues, 2007). Lactic acid and acetic acid are the most frequently used organic acids for this purpose; however, 1% fumaric acid has been found to be more effective than 1% lactic or acetic acid against *S. enterica* Typhimurium, *L. monocytogenes*, and *Escherichia coli* (Podolak et al., 1995b). An organic acid already used in animal diets that is similar in structure to fumaric acid is methionine hydroxy analogue (MHA) (Dibner and Buttin, 2002).

MHA is a methionine supplement available in liquid format, Alimet® and is commonly used as an alternative to anhydrous DL-methionine (DLM) to improve protein quality of swine and poultry diets. Currently, DLM is often included in commercial feline extruded and canned diets since methionine is the first limiting amino acid in animal-based diets (Klemesrud et al., 1997), and cats, as obligate carnivores, have a higher requirement for sulfur-containing amino acids compared to omnivorous animals (MacDonald and Rogers, 1984). Since the mixing of ingredients in RMBDs introduces the contamination from the surface of the meat to the interior and throughout the diet, liquid Alimet® may combat this problem better than anhydrous DLM.

The effectiveness of Alimet® against *Salmonella* spp. or *L. monocytogenes* had not been tested before, therefore the first aim of this study was to determine the minimum concentration of Alimet® needed to inhibit growth of *S. Typhimurium* and *L. monocytogenes* under normal
laboratory growth conditions. After establishing that Alimet® was effective against these pathogens under normal growth conditions, the next goal of the study was to assess the effectiveness of Alimet® at inhibiting bacterial growth in raw meat inoculated with cocktails of *S. enterica* and *L. monocytogenes* and stored by freezing then thawing in a refrigerator. We hypothesized that less than 1% Alimet® would inhibit growth of *S. Typhimurium* and *L. monocytogenes* in nutrient broth under ideal growth conditions, and that Alimet® included as 1% of the sample would inhibit growth of *S. enterica* and *L. monocytogenes* in raw meat after freezing, then thawing in a refrigerator for up to 72 hours.

6.3. Materials and methods

6.3.1. Minimum inhibitory concentration

To determine the minimum inhibitory concentration (MIC) of Alimet® needed to stop growth of *S. Typhimurium* and *L. monocytogenes*, a specialized plate reader (Bioscreen C MBR, Oy Growth Curves Ab Ltd, Helsinki, Finland) was used to measure changes in optical density over time when plate wells containing bacteria culture were challenged with ten Alimet® (Novus International, Saint Charles MO, USA) concentrations in tryptic soy broth (TSB; Beckton, Dickinson, and Company, Sparks, MD, USA) ranging from 0.10 – 1.00% Alimet® (v/v).

Treatment preparations

Alimet® and TSB stock solutions were made in advance to avoid pipetting less than 50μL, then heated in a hot water bath at 60.0°C for 24 hours to facilitate even mixing, then vortexed and kept at room temperature for 24 hours prior to the experiment to avoid temperature bias. Final concentrations of Alimet® in the Bioscreen wells were 0.10 – 1.00% v/v. Sterile TSB
containing no Alimet® (0.00%) was used as a control treatment to demonstrate normal bacterial growth.

**Treatment pH measurements**

A pH metre (symPHony B10P, VWR, Radnor PA, USA) was used to determine the pH of the final solution used in the Bioscreen wells. The metre was calibrated according to the manufacturer’s instructions using a neutral (pH 7.0) and an acidic (pH 4.0) solution. To mimic the solution in the Bioscreen wells, 2 mL of each treatment solution was added to a glass test tube containing 1 mL of phosphate buffer solution (PBS; Beckton, Dickinson, and Company, Sparks MD, USA). Each tube was vortexted for 10 seconds, then the pH probe was inserted into the tube so that the electrode was immersed in the liquid. Readings were taken until three identical measurements were recorded for each solution.

**Culture preparation**

*Salmonella enterica* subsp. Typhimurium (ATCC 700408; supplied by the Public Health Agency of Canada, Guelph, ON, Canada) and *Listeria monocytogenes* (ATCC 19115; supplied by University of Guelph Laboratory Services, Guelph ON, Canada) were streaked from frozen culture onto plates of tryptic soy agar (TSA; Beckton, Dickinson, and Company, Sparks MD, USA) and incubated at 37.0°C for 24 hours (HeraTherm Refrigerated Incubator, ThermoScientific, Waltham MA, USA). For each species, one colony was then transferred to 10mL of TSB and incubated for 24 hours at 37.0°C while shaking at 180 rpm (Innova 42R, New Brunswick Scientific, Enfield CT, USA). The inoculated tubes were then washed twice in phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis MO, USA) at 4000xg (Sorvall ST16R Centrifuge, Thermo Scientific, Waltham MA, USA) for 10 minutes then resuspended in 10mL of
PBS. Next, the bacterial cultures were standardized in fresh PBS using a cell density metre (model 40, Fisher Scientific, Waltham MA, USA) and McFarland standard (McFarland Equivalence Turbidity Standard 0.5, ref R20410; Remel, Lenexa KS, USA). The optical density of McFarland standard no. 0.5 is expected to equate to an approximate cell density of $1.5 \times 10^8$ CFU/mL (Remel, 2009).

**Optical density measurement**

A 100-well plate (Honeycomb, Oy Growth Curves Ab Ltd, Helsinki, Finland) was prepared for each bacterial species. This was done by adding 200μL of the appropriate Alimet®-treated TSB and 100μL of the standardized bacterial solution to each well. One column of wells contained 100μL of sterile PBS and 200μL of TSB per well, to determine the absorbance of these solutions without the presence of bacteria or Alimet® (control blanks). Three subsequent columns contained 100μL of sterile PBS and 200μL of Alimet®-treated TSB per well, with one concentration per row, to determine the optical density of these solutions without the presence of bacteria (treatment blanks). One column was left empty to provide a buffer zone between uninoculated (blanks) and inoculated wells. The next three columns were filled with 100μL of standardized bacterial cultures and 200μL of the respective Alimet® solutions per well (treatments). One column was left empty to give a buffer zone between treated and untreated bacterial cultures. The last column contained 100μL of inoculated PBS and 200μL of untreated TSB per well (controls). One plate was prepared for each species and the two plates were placed in the reader at the same time. The reader was set at a wavelength of 600nm and absorbance measurements were taken every 30 minutes over 48 hours at 30°C and gently shaking the plates for 30 seconds before taking each measurement to resuspend settled cells. After 48 hours when the run was complete, the process was repeated twice more, such that the experiment was
repeated in triplicate. Following the completion of each Bioscreen run, inoculating loops were used to transfer the solution from each Alimet®-and-bacteria-containing well to a TSA plate, that was then incubated at 37.0°C for 24 hours to allow surviving bacteria to colonize the media, indicating if the Alimet® treatment had been bactericidal or bacteriostatic.

**Bacterial growth curves**

All optical density (OD) data for the controls was averaged and normalized by subtracting the average OD of the control blanks, to ensure only the absorbance of the bacteria was considered, not the absorbance from PBS and TSB. All OD data for the treatments were averaged and normalized by subtracting the average OD of the treatment blanks, to ensure only the absorbance of the bacteria was considered, not the absorbance from PBS, TSB, and Alimet®. This was repeated for each plate and the three plates for each species were averaged. Average optical density data for the treatments and control were plotted over time to produce bacterial growth curves for each species.

**6.3.2. Efficacy in meat**

To assess the efficacy of Alimet® as an antimicrobial agent in RMBDs, batches of raw ground meat were contaminated with 5-strain cocktails of either *S. enterica* or *L. monocytogenes* and challenged with four concentrations of Alimet®, then sampled for viable bacteria following a 24-hour freeze and being thawed in a refrigerator for 24, 48, and 72 hours.

**Inoculum preparation**

Five strains of *S. enterica* (ATCC13076, ATCC14028, ATCC8326, and ATCC6962 supplied by the University of Guelph Lab Services, Guelph, ON, Canada, and SAI20150301 supplied by the Public Health Agency of Canada, Ottawa, ON, Canada) and five strains of *L.
monocytogenes (LI0512, LI0529, ATCC19115, ATCC19111 supplied by the University of Guelph Lab Services, Guelph, ON, Canada, and 08-5578 supplied by the National Microbiology Lab, Winnipeg, MN, Canada) were selected for use in this experiment. These particular strains were chosen as they had all been isolated from beef or chicken. All bacteria were stored at -80°C until needed. Four days before the setup date (day 0), each strain was streaked onto a tryptic soy agar (TSA; Beckton, Dickinson, and Company, Sparks MD, USA) plate from the frozen stock, then incubated at 37.0°C for 24 hours (HeraTherm Refrigerated Incubator, ThermoScientific, Waltham MA, USA). Following incubation, a single colony was transferred from each plate to its own tube containing 10 mL of tryptic soy broth (TSB; Beckton, Dickinson, and Company, Sparks MD, USA) and incubated for 24 hours at 37.0°C while shaking at 180 rpm (Innova 42R, New Brunswick Scientific, Enfield CT, USA). To acid-adapt each strain, 10μL of inoculated TSB was transferred to a tube of 10 mL of TSB containing 1% glucose (α-D-glucose 96%, Alrich Chemical Company, Millwauke WI, USA) and incubated for 24 hours at 37.0°C while shaking at 180 rpm. Finally, for each strain, 100μL of inoculated TSB+1.0% glucose was transferred to a fresh tube, containing 10mL of TSB+1.0% glucose. This tube was incubated at 37.0°C while shaking at 180 rpm for 24 hours.

Cocktail preparation

For each setup, a cocktail was prepared by adding 0.5mL of inoculated TSB+1.0% glucose of each strain (2.5mL total) to a tube containing 7.5mL of phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis MO, USA). These tubes were centrifuged at 4000xg (Sorvall ST16R Centrifuge, Thermo Scientific, Waltham MA, USA) for 10 minutes to form a cell pellet. The supernatant was then discarded, and cells were resuspended in 10mL of PBS. This preparation procedure was intended to yield approximately 2.5x10^5 CFU per batch (2.5kg) of
meat. One cocktail tube was made to contaminate the meat and an identical one was made to determine the bacterial concentration of the inoculum.

**Meat preparation**

Regular lean ground beef and extra lean ground chicken were obtained from a local grocery store in Guelph, ON, Canada. A total of six batches of meat were prepared for the study, with each batch weighing 2.5kg. Three batches were inoculated with the cocktail of *S. enterica* strains and three batches were inoculated with the cocktail of *L. monocytogenes*. Prior to inoculation, 1.25kg of ground beef and 1.25kg of ground chicken were thoroughly combined in a mixer (Cuisinart SM-70BCC, Stamford CT, USA). The cocktail of either *S. enterica* or *L. monocytogenes* was then added to the meat and thoroughly mixed again.

**Meat treatment**

After inoculation, the meat mixture was divided into five portions of 500g. Each portion received one of the five treatments: 0.00% (control), 0.50%, 0.75%, 1.00%, and 1.25% Alimet® on a w/w basis. To accomplish this, the quantity of meat was weighed out minus the weight of Alimet being added such that the total final weight of the sample was 500g when Alimet® was added, and then the Alimet® and meat were mixed thoroughly by hand. Once Alimet® was evenly distributed, each portion of meat was separated into four sub-portions of 113g and formed into a patty (Starfrit Patty Stacker, Atlantic Promotions, Longueuil QC, Canada.). Patties were then individually vacuum-packaged (C-200, Multivac, Kansas City MO, USA) and one for each treatment was kept at room temperature for determination of starting contamination levels as described below. The other three patties for each treatment were placed in a freezer (model V05NAA, Wood’s, Guelph ON, Canada) and frozen at -20.0°C. They remained in the freezer for
24 hours, then were relocated to a fridge (model FFU20F9GW3, Frigidaire, Charlotte NC, USA) to thaw at 4.0°C. For each treatment, one patty was removed after thawing in the refrigerator for 24 hours, one after 48 hours, and one after 72 hours. Once patties were removed from the fridge their contamination levels were determined as described below.

**Cocktail colony count determination**

Colony counts were determined for the *S. enterica* and *L. monocytogenes* cocktails that were used to inoculate the meat for each trial. This was done using either XLT4 agar (Difco XLT4 base and supplement, Beckton, Dickinson, and Company, Sparks MD, USA), a selective growth media for *S. enterica*, or Oxford agar (Difco Oxford medium base, Beckton, Dickinson, and Company, Sparks MD, USA; Oxford-listeria-selective supplement 1.07006.0010, Merck KGaA, Billerica MA, USA), a selective growth media for *L. monocytogenes*. To do this, 100μL of each 10mL cocktail was added to a microcentrifuge tube containing 900μL of 0.1% peptone water; this generated a ten-fold dilution. From that ten-fold dilution ($10^{-1}$), 100μL was added to a fresh microcentrifuge tube containing 900μL of 0.1% peptone water; this generated a 100-fold dilution ($10^{-2}$). These serial dilutions were repeated until there were dilutions from $10^{-1}$ to $10^{-6}$. Each dilution was plated in duplicate by pipetting 100μL onto a selective growth media plate and evenly distributing the solution across the plate with an L-shaped cell spreader (Fisher Scientific, Waltham MA, USA). The plates were then incubated at 37.0°C for 24 hours (model MIR-553, Sanyo, Osaka, Japan). Yellow-ringed black colonies that formed on the XLT4 plates indicated growth of the *S. enterica* strains, and black-ringed white colonies that formed on the Oxford plates indicated growth of the *L. monocytogenes* strains. For each sample, the two plates for the dilution which had produced between 25 and 250 colonies were counted. These counts were
averaged, multiplied by the dilution factor, and divided by the quantity of the cocktail to determine the contamination level of the cocktail (CFU/mL).

**Sample colony count, water activity, and pH determination**

At each respective timepoint (time 0, 24h-thaw, 48h-thaw, and 72h-thaw), patties were removed from refrigerator (if applicable) for sampling. Each vacuum package was sliced open and 7g was allocated to a small plastic cup for pH measurement (symPHony SP70P, VWR, Radnor PA, USA) followed by water activity determination (AquaLab 4TE, Decagon Devices, Pullman WA, USA). For colony count determination, 25g of the meat sample was added to a stomacher sampling bag (VWR, Rador PA, USA), along with 225g of 0.1% peptone water (Beckton, Dickinson, and Company, Sparks MD, USA). The contents of the sampling bag were blended by a stomacher (Stomacher® 400 Circulator, Seward Laboratory Systems Inc, Islandia NY, USA) and the bag’s filtration system allowed for the collection of a liquid sample which was a 10-fold dilution of the 25g sample ($10^{-1}$). Next, three serial dilutions were made from each stomached sample and 100μL from each dilution (including the stomached sample) was pipetted onto the appropriate agar plate and the solution was spread across the plate with an L-shaped spreader. Contamination levels were determined by counting all colonies on each plate from the dilution that had yielded between 25 and 250 colonies and taking the average of the two plates for that dilution. When required, to obtain colony counts for the lowest detection limit, 1mL of stomached sample was transferred to four agar plates (250μL on each plate) and the solution on each plate was spread with an L-shaped spreader. Contamination levels could be determined for this dilution by adding the colony counts of the four plates together, then averaging them with the total count from the duplicate set.
**Log reduction calculations**

Average colony counts for each sample were multiplied by their dilution factor, divided by the amount of sample plated (mL), and divided by the weight of sample used (g), to determine the number of colony-forming units (CFU) per gram of sample. This value for each sample was then log-transformed to determine logCFU/g of each sample. The log reduction of each treatment was determined by subtracting the logCFU/g of each treatment on day 0 from the logCFU/g of that treatment at each timepoint. This was graphed over time to show the reduction of bacteria over time for each treatment.

**6.3.3. Statistical analyses**

Optical density data from the Bioscreen runs were analyzed using the mixed model procedure of a commercial software (SAS version 9.4; SAS Institute, Cary NC, USA). Normalized optical density variables were separated by bacteria species and run-date. These variables were analyzed using repeated measures over time in a mixed model with Alimet® concentration, time, and the interaction of concentration and time as the fixed effects. Data were reduced to optical density every six hours rather than every 30 minutes as this was more data points than necessary for the program. The pH variables from these experiments were analyzed in a mixed model using concentration as the fixed effect and run as the random effect. The area under the curve (AUC) for each Alimet® concentration against each species was calculated using the trapezoidal method and these data were analyzed using a mixed model with concentration as a fixed effect and run as the random effect. Differences were considered significant at $P \leq 0.05$ and indicated bacterial growth had occurred.
Colony count data from the meat trials were analyzed by using the generalized linear mixed model procedure of a commercial software (SAS version 9.4; SAS Institute, Cary, NC). Log-transformed colony count variables (logCFU/g) were analyzed separately by bacteria species. These variables were analyzed in a mixed model with Alimet® concentration, time, and the interaction of concentration and time as the fixed effects, and batch as the random effect. Differences were considered significant at $P \leq 0.05$ and indicated a decline in bacterial growth. The pH variables from these experiments were analyzed in a mixed model using concentration and the interaction of concentration and time as the fixed effect, and batch as the random effect. Differences were considered significant at $P \leq 0.05$.

6.4. Results

6.4.1. Minimum inhibitory concentration

Mean pH values for the solutions in the Bioscreen wells are graphed in relation to the final concentration of Alimet® in Figure 6.1., and pH decreased as the concentration of Alimet® increased, as was expected. The changes in OD over time for each treatment against $S$. Typhimurium and $L. monocytogenes$ are presented in Figure 6.2. and 6.3., respectively. As expected, the OD of the control wells containing no Alimet® increased over time in the cases of both $S$. Typhimurium and $L. monocytogenes$ ($P<0.0001$, $P<0.0001$), indicating that bacterial growth did occur. The OD of the wells containing 0.1% Alimet® and 0.2% Alimet® also increased over time in the cases of both $S$. Typhimurium and $L. monocytogenes$ ($P<0.0001$, $P<0.0001$), indicating that these bacteria continued to grow in the presence of these concentrations of Alimet®. The OD of the wells containing 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, and 1.0% Alimet® did not change over time ($P>0.05$, $P>0.05$), indicating that bacteria could not grow in the presence ≥0.3% Alimet®. For both $S$. Typhimurium and $L.$
monocytogenes, there was a difference in the AUC of the control and the 0.1% and 0.2% Alimet® treatments, but no difference in the AUC of the 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, and 1.0% Alimet® treatments. Based on these findings, it was determined that MIC of Alimet® against S. Typhimurium was 0.3%, and that the MIC of Alimet® against L. monocytogenes was also 0.3%. When Alimet®-treated well solutions were plated on TSA, S. Typhimurium colonies formed from the 0.1% and 0.2% Alimet®-treated solutions as expected, and no colonies formed from 0.3% or higher treatment solutions. For L. monocytogenes, colonies formed from the 0.1% and 0.2% Alimet®-treated solutions, as well as from the 0.3% and 0.4% Alimet®-treated solutions, but not from 0.5% or higher treatment solutions.

Figure 6.1. Mean pH values of solutions in Bioscreen wells based on final Alimet® concentrations.
Figure 6.2. Growth of *Salmonella enterica* Typhimurium DT104 at 30.0°C in TSB mixed with different amounts of Alimet®. Final concentrations of Alimet® in wells were 0.0% (control) 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1.0%. Y-axis OD600 normalized for absorbance of wells without bacterial growth.
Figure 6.3. Growth of *Listeria monocytogenes* ATCC 19115 at 30.0°C in TSB mixed with different amounts of Alimet®. Final concentrations of Alimet in wells were 0.0% (control) 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1.0%. Y-axis OD600 normalized for absorbance of wells without bacterial growth.

6.4.2. Efficacy in meat

The mean pH values of the samples inoculated with *S. enterica* are given in Table 6.1. and the mean pH values of the samples inoculated with *L. monocytogenes* are given in Table 6.2. For both inoculum, all Alimet®-treated samples had pH values that were lower than the control ($P<0.0001$, $P<0.0001$), the overall mean pH was different among all treatments ($P<0.0001$, $P<0.0001$), and pH did not differ within each treatment over time ($P>0.05$, $P>0.05$).
Table 6.1. Average pH values (mean±s.e.m.) from three batches of raw ground meat (50% beef, 50% chicken) samples inoculated with *Salmonella enterica* and mixed with different quantities of Alimet®, tested immediately or frozen at -20.0°C for 24 hours, then kept in a refrigerator at 4.0°C and tested 24, 48, and 72 hours after removal from freezer. Alimet® constituted 1.25%, 1.00%, 0.75%, 0.50%, and 0.00% (control) of each sample.

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Least square means within a row not sharing a common superscript letter differ (*P*<0.05), p-value refers to the analysis of variance of the effects of treatment.
Table 6.2. Average pH values (mean±s.e.m.) from three batches of raw ground meat (50% beef, 50% chicken) samples inoculated with *Listeria monocytogenes* and mixed with different quantities of Alimet®, tested immediately or frozen at -20.0°C for 24 hours, then kept in a refrigerator at 4.0°C and tested 24, 48, and 72 hours after removal from freezer. Alimet® constituted 1.25%, 1.00%, 0.75%, 0.50%, and 0.00% (control) of each sample.

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Least square means within a row not sharing a common superscript letter differ (*P*≤0.05), p-value refers to the analysis of variance of the effects of treatment.

Colony count data for *S. enterica* from the three meat mixture batches is presented in Table 6.3. as the mean log-transformed CFU/g (logCFU/g). When comparing the *S. enterica* contamination levels for treatments in contrast to the control on a same-day basis, no concentration of Alimet® demonstrated any effect on *S. enterica* compared to the control on day 0 or day 3 (*P*>0.05). On day 2, there was 20% less *S. enterica* (0.67 log difference) in the 1.25% Alimet® sample compared to the control (*P*=0.0003), and no difference between the control and the other Alimet® treatments (*P*>0.05). On day 4, there was 17% less *S. enterica* (0.51 log difference) in the 1.25% Alimet® samples compared to the control (*P*=0.0043), and no difference between the control and the other Alimet® treatments (*P*>0.05).
Table 6.3. Average log-transformed colony count data (mean±s.e.m.; logCFU/g) for *Salmonella enterica* in inoculated raw ground meat (50% beef, 50% chicken) mixed with different quantities of Alimet®, tested immediately or frozen at -20.0°C for 24 hours, then kept in a refrigerator at 4.0°C and tested 24, 48, and 72 hours after removal from freezer. Alimet® constituted 1.25%, 1.00%, 0.75%, 0.50%, and 0.00% (control) of each sample.

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\textsuperscript{a,b} Least square means within a row not sharing a common superscript letter differ (\(P\leq0.05\)), p-value refers to the analysis of variance of the effects of treatment.

Colony count data for *L. monocytogenes* from the three meat mixture batches is presented in Table 6.4. as the mean log-transformed CFU/g (logCFU/g). When comparing *L. monocytogenes* contamination levels of treatments in contrast to the control on a same-day basis, there was no difference between samples treated with Alimet® compared to the controls on day 0 (\(P>0.05\)). On day 2, there was 15%, 21%, and 26% less *L. monocytogenes* (0.98, 0.92, and 1.14 log differences) in the 0.75%, 1.00%, and 1.25% Alimet® treatment samples, respectively, compared to the control samples (\(P=0.0402, P<0.0001\), and \(P<0.0001\)). On day 3, there was 11% and 16% less *L. monocytogenes* (0.47 and 0.71 log difference) in the 1.00% and 1.25% Alimet® treated samples, respectively, compared to the control (\(P=0.0034\) and \(P<0.0001\)). On day 4, there
was 10% and 17% less *L. monocytogenes* (0.45 and 0.76 log difference) in the 1.00% and 1.25% Alimet®-treated samples, respectively, compared to the control (*P*=0.0055 and *P*<0.0001).

Alimet® included as 0.50% of the sample did not have an effect on *L. monocytogenes* compared to the control on any days (*P*>0.05).

Table 6.4. Average log-transformed colony count data (mean±s.e.m.; logCFU/g) for *Listeria monocytogenes* in inoculated raw ground meat (50% beef, 50% chicken) mixed with different quantities of Alimet®, tested immediately or frozen at -20.0°C for 24 hours, then kept in a refrigerator at 4.0°C and tested 24, 48, and 72 hours after removal from freezer. Alimet® constituted 1.25%, 1.00%, 0.75%, 0.50%, and 0.00% (control) of each sample.

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a, b, c Least square means within a row not sharing a common superscript letter differ (*P*≤0.05), p-value refers to the analysis of variance of the effects of treatment.

The mean log reductions of *S. enterica* and *L. monocytogenes* in raw ground meat within treatments over time are shown in Figure 6.4. and Figure 6.5., respectively. Within the control samples, *S. enterica* decreased by 18% from day 0 to 2 (0.78 log reduction; *P*<0.0001), decreased by 27% from day 0 to 3 (1.12 log reduction; *P*<0.0001), and decreased by 28% from day 0 to 4 (1.18 log reduction; *P*<0.0001). When comparing the control samples from one day to the next, *S. enterica* decreased by 10% from day 2 to 3 (0.35 log reduction; *P*=0.049), and there
was no difference from day 3 to 4 ($P$>0.05). Within the 0.50% Alimet®-treated samples, *S. enterica* decreased by 18% from day 0 to 2 (0.75 log reduction; $P$<0.0001), decreased by 19% from day 0 to 3 (0.77 log reduction; $P$<0.0001), and decreased by 19% from day 0 to 4 (0.80 log reduction; $P$<0.0001). Next, within the 0.75% Alimet®-treated samples, *S. enterica* decreased by 17% from day 0 to 2 (0.69 log reduction; $P$=0.0002), decreased by 20% from day 0 to 3 (0.78 log reduction; $P$<0.0001), and decreased by 17% from day 0 to 4 (0.67 log reduction; $P$=0.0003). After that, within the 1.00% Alimet®-treated samples, *S. enterica* decreased by 20% from day 0 to 2 (0.82 log reduction; $P$<0.0001), decreased by 25% from day 0 to 3 (1.01 log reduction; $P$<0.0001), and decreased by 23% from day 0 to 4 (0.94 log reduction; $P$<0.0001). Finally, within the 1.25% Alimet®-treated samples, *S. enterica* decreased by 30% from day 0 to 2 (1.20 log reduction; $P$<0.0001), decreased by 30% from day 0 to 3 (1.21 log reduction; $P$<0.0001), and decreased by 37% from day 0 to 4 (1.44 log reduction; $P$<0.0001). When comparing within the 0.50%, 0.75%, 1.00%, and 1.25% Alimet® samples from one day to the next, there was no difference from day 2 to 3 or day 3 to 4 ($P$>0.05).
Fig. 6.4. Log reduction of *Salmonella enterica* in inoculated raw ground meat (50% beef, 50% chicken) mixed with different quantities of Alimet®, tested immediately or frozen at -20.0°C for 24 hours, then kept in a refrigerator at 4.0°C and tested 24, 48, and 72 hours after removal from freezer. Alimet® constituted 1.25%, 1.00%, 0.75%, 0.50%, and 0.00% (control) of each sample.

For *L. monocytogenes*-inoculated samples, there was no difference in the contamination levels of *L. monocytogenes* found over time within treatment groups for the control samples or the 0.50% Alimet®-treated samples (*P*>0.05). Within the 0.75% Alimet®-treated samples, *L. monocytogenes* decreased by 13% from day 0 to 2 (0.58 log reduction; *P*<0.0001), and did not differ between day 0 and 3, or day 0 and 4 (*P*>0.05). When comparing from one day to the next, *L. monocytogenes* levels were 10% higher (0.36 log difference; *P*=0.0248) in the 0.75% Alimet® treatment on day 3 compared to day 2. Next, within the 1.00% Alimet®-treated samples, *L. monocytogenes* decreased by 18% from day 0 to 2 (0.81 log reduction; *P*<0.0001), was 9% lower on day 3 compared to day 0 (0.40 log difference; *P*=0.0113), and was 8% lower on day 4 compared to day 0 (0.36 log difference; *P*=0.0234). Also, when comparing from one day to the next, *L. monocytogenes* levels were 12% higher (0.40 log difference; *P*=0.0116) in the 1.00% Alimet® treatment on day 3 compared to day 2. Finally, within the 1.25% Alimet®-treated samples, *L. monocytogenes* decreased by 21% from day 0 to 2 (0.85 log reduction; *P*<0.0001), was 11% lower on day 3 compared to day 0 (0.47 log difference; *P*<0.0035), and was 12% lower on day 4 compared to day 0 (0.51 log difference; *P*<0.0019). In addition, when comparing from one day to the next, *L. monocytogenes* levels were 14% higher (0.38 log difference; *P*<0.0167) in the 1.25% Alimet® treatment on day 3 compared to day 2.
Fig. 6.5. Log reduction of *Listeria monocytogenes* in inoculated raw ground meat (50% beef, 50% chicken) mixed with different quantities of Alimet®, tested immediately or frozen at -20.0°C for 24 hours, then kept in a refrigerator at 4.0°C and tested 24, 48, and 72 hours after removal from freezer. Alimet® constituted 1.25%, 1.00%, 0.75%, 0.50%, and 0.00% (control) of each sample.

**6.5. Discussion**

This was the first study to investigate the MIC of Alimet® against *S. Typhimurium* and *L. monocytogenes* under normal laboratory growth conditions, as well as the effectiveness of Alimet® against these pathogens in a more complex environment, raw ground meat. The MIC of Alimet® against both *S. enterica* and *L. monocytogenes* was 0.3%, which was lower than expected; however, Alimet® was not bactericidal against *L. monocytogenes* until 0.5%. In ground meat, Alimet® included as 1.25% of the sample reduced both *S. enterica* and *L. monocytogenes* growth compared to the control; however, these pathogens never fell below detection levels as had been hypothesized.
Finding the minimum inhibitory concentration of Alimet® to be the same against *S. enterica* and *L. monocytogenes* was not expected; however, a closer inspection of the bacterial growth curves revealed that there were differences between the responses of the two species. *L. monocytogenes* reached peak growth in 12 hours and optical density remained relatively stable for the remainder of the experiment. On the other hand, *S. Typhimurium* first appeared to have reached peak growth in 18 hours, but after another 18 hours had passed, at the 36-hour mark, *S. Typhimurium* began to demonstrate growth again that continued up to the end of the experiment, which was not expected. As a Gram-positive bacteria species, *L. monocytogenes* is better equipped to handle an acidic environment than a Gram-negative bacteria species such as *S. Typhimurium* (Ramos et al., 2001; Jordan et al., 2008), which explains why *L. monocytogenes* reached peak growth faster than *S. Typhimurium*. In addition, the optical density of untreated *L. monocytogenes* at its peak time (12 hours) was 0.5884, whereas the optical density of untreated *S. Typhimurium* at its peak time (18 hours) was 0.4675, demonstrating that *L. monocytogenes* not only grew faster, but also had greater growth in this period. However, this is confounded by the second unexpected growth phase that *S. Typhimurium* experienced.

Prior to this study, it had been established that 1% MHA and 1% formic acid were equally effective when corrected to pH 4.0 against $10^6$ CFU of *E. coli* in tryptic soy nutrient broth, resulting in total bacteriolysis after 24 hours (Dibner and Buttin, 2002). Although pH is hypothesized to be the primary factor that determines the effectiveness of organic acids against bacteria (Carpenter and Broadbent, 2009), the fact that 1% lactic acid corrected to pH 4.0 was not as effective as MHA and formic acid against *E. coli* in the Dibner & Buttin (2002) study suggests that there are other factors that contribute to the effectiveness of organic acids as antimicrobials, such as their dissociation constant ($pK_a$), which indicates the strength of the acid.
(Cherrington et al., 1991). In the current study, 0.3% Alimet® (mean pH 4.79) was bactericidal against approximately $1.5 \times 10^7$ CFU of S. Typhimurium and 0.5% Alimet® (mean pH 4.05) was bactericidal against approximately $1.5 \times 10^7$ CFU of L. monocytogenes. A direct comparison of these results to those of the Dibner & Buttin (2002) suggests that Alimet® is more effective against L. monocytogenes and S. enterica than E. coli; however, differences in experimental design and protocols may account for some of this. Given that the mean pH of the 1% Alimet® solution in the current study was 3.49, this suggests that the pH of 1% MHA solution in the Dibner & Buttin (2002) study likely had to be raised using NaOH. If this was the case then the two studies should be compared on a pH basis, rather than a concentration basis and Alimet® would appear to be equally effective against L. monocytogenes and only slightly more effective against S. Typhimurium compared to E. coli. However, this cannot be said for certain as the effectiveness of the organic acids in the Dibner & Buttin (2002) study were not tested at other pHs. Therefore, Alimet® is at least as effective against S. Typhimurium and L. monocytogenes as it is against E. coli under normal laboratory growth conditions.

While Alimet® did exhibit some effect against both S. enterica and L. monocytogenes when included as 1.25% of the ground meat sample, Alimet® was less effective against S. enterica and L. monocytogenes in ground meat than we had anticipated. We had hypothesized that Alimet® included as 1.00% of the sample would reduce pathogens below the detection limit; however, this did not occur with either pathogen, even when Alimet® was included as 1.25% of the sample. These results suggest that the ground meat environment was substantially more favourable for the growth and protection of bacteria than we had anticipated.

As expected, there was clear variability between the responses of the two bacteria species to the experiment as was observed in the control samples. S. enterica was reduced in the control
treatment over time from day 0 to day 2, during which the samples were frozen for 24 hours, then defrosted in a refrigerator for the next 24 hours. This would suggest that the handling and storage of raw diets that are contaminated with *S. enterica* might be an important hazard control measure to consider, especially since *S. enterica* continued to decline over the duration of the experiment when the samples remained in the refrigerator for 48 and 72 hours after being removed from the freezer. However, this requires further investigation, especially since these findings were contradictory to those of other studies which have investigated the effects of defrosting on pathogens in raw ground meat. Manios & Skandamis (2015), contaminated ground beef with 6.8 logs of *S. enterica*, froze their beef patties at -22°C for 5 days, then thawed in a refrigerator at 4°C for 16h, and did not observe a difference in contamination levels of the beef patties before and after defrosting. Similarly, Lianou & Koutsoumanis (2009) contaminated ground beef with 7.15 logs of *S. enterica*, froze their samples at -25°C for 3 days, then thawed them in a refrigerator at 5°C for 15h, and did not observe a difference in contamination levels before and after defrosting. Several differences between our experiment and theirs may explain some of the discrepancy between our results, such as their higher initial contamination levels, the use of only beef, longer freeze times, shorter thaws times, the use of different strains of *S. enterica*, and the use of different selective media for growth and numeration.

One fact that might reduce the likelihood that the above-mentioned factors contributed to the difference in results though is that Lianou & Koutsoumanis (2009) also tested *L. monocytogenes* in their study using the same parameters, and like us, saw no difference in *L. monocytogenes* contamination levels before and after defrosting. This may have more to do with the resilience of *L. monocytogenes* though, than the testing parameters, as the cell wall of Gram-
positive bacteria are generally regarded to be more robust and better able to handle environmental stressors than the cell envelope of Gram-negative bacteria (Hurst, 1977).

In terms of the effect of the Alimet® treatments on pathogens in contrast to the control samples on a same day basis, 1.25% Alimet® was the only treatment found to have an effect that was common to both pathogens, on all days for *L. monocytogenes* and on days 2 and 4 for *S. enterica*. We can fairly confidently propose that the ability of *L. monocytogenes* to grow at temperatures as low as 2°C (Gandhi and Chikindas, 2007) contributed to the larger number of statistically significant results found when comparing levels of *L. monocytogenes* in the Alimet®-treated samples to the control samples. While 1.25% Alimet® affected *L. monocytogenes* contamination levels compared to the control on all days, it is of note that these levels had reached their lowest point on day 2 (after 24 hour freeze, followed by 24 hour thaw in refrigerator), then demonstrated a significant increase from day 2 to 3, and no change from day 3 to 4, though these levels were still significantly different compared to the control, which had not changed. For samples inoculated with *S. enterica*, no difference in contamination levels was identified within any treatment after day 2; however, it is noteworthy that the difference between levels of *S. enterica* in the control and 1.25% Alimet® treatment were no longer statistically different on day 3, though they were lower in the 1.25% Alimet® treatment again on day 4. This growth of *L. monocytogenes* between day 2 and 3, and lack of difference between *S. enterica* levels in the control and 1.25% Alimet® treatment on day 3, both further support recommendations already made by some commercial raw pet food manufacturers for pet owners to feed raw pet food within 24 hours of removal from freezer.

Finally, when comparing the results of this study to those of Hasty (2017) when investigating the efficacy of HPP against bacteria in an inoculated raw pet diet, we can again
take note of some differences between their experiment and ours, and question the impact these differences may have had on our results. For their experiment, a beef diet inoculated with 6.7 logs (based on counts from selective media immediately following inoculation) of non-pathogenic *E. coli* strains (representative of pathogenic *S. enterica*), then was processed by HPP and either sampled within 24h or frozen at -23°C for 5 days then sampled. They observed a 4.9 log reduction in the samples tested immediately following HPP, and a 6.2 log reduction with 90% of samples below detection limits from samples frozen for 5 days after HPP treatment.

Given that this study used only Gram-negative bacteria, it is not surprising to see that freezing had such a big effect on bacteria survival, as was also seen in our study with *S. enterica*. Since their study had much higher initial contamination levels, we can try to compare their results with ours on a proportional basis. In that case, Hasty (2017) observed *E. coli* contamination levels fall by 73% following HPP, which is a greater difference than both the 30% decrease observed in *S. enterica* levels and the 21% decrease observed in *L. monocytogenes* levels when comparing initial contamination levels to contamination levels on day 2 (frozen for 24h then thawed in refrigerator for 24h) in samples containing 1.25% Alimet®. While HPP alone may be a more effective treatment than 1.25% Alimet® in a raw diet, HPP did not result in total kill in all samples even when samples were frozen for 5 days, and HPP may not be available to all raw pet food manufacturers. As such, since Alimet® was found to have an effect on pathogens, it warrants further investigation to determine if a higher concentration of Alimet® can result in total kill of pathogens in raw ground meat, as well as whether other hazard control technologies such as freezing duration and temperature can be used in conjunction with similar concentrations of Alimet® to mitigate bacterial contamination in raw diets for pets.
Finally, it also must be determined if raw diets with 1.25% Alimet® are palatable and accepted by cats, as this would be a key factor in determining the applicability of this work to commercial raw diet manufacturers, in addition to Alimet® improving protein quality (Shoveller et al., 2010; Zimmermann et al., 2005). As well, acidifying feline diets with Alimet® may prevent the formation of urinary struvite crystals, a common problem among cats (Skoch et al., 1991); however, over acidification could cause an imbalance to the cat’s acid-base balance therefore this should also be monitored.

6.5.1. Conclusions

Under laboratory testing conditions, 0.5% Alimet® was bactericidal against *S.* Typhimurium and *L. monocytogenes*. Alimet® included as 1.25% of a raw meat mixture reduced *S. enterica* and *L. monocytogenes* contamination raw meat by 30% and 20% when meat samples were frozen (-20°C, 24h), then thawed in a refrigerator (4°C, 24h); however, 1.25% Alimet® did not result in total kill of pathogens in raw ground meat. Further investigation into the usefulness of Alimet® as an antimicrobial agent, among its other properties, in raw pet diets is warranted.
7. General Discussion

Investigating ways to improve existing diets for animals is one of the fundamental aspects of the study of animal nutrition, and as such, the overall objective of this thesis was to assess the effects of various treatments on diets for companion animals. By modifying current diets, animal nutritionists can introduce changes that can be more easily employed by feed manufacturers and animal caretakers. When more than one method is found to tackle a certain issue with a diet, scientists can study how these methods may affect other features in the diet and how these changes impact the animal consuming the diet to determine the benefits and drawbacks of each method. This was the case in the first study of this thesis, in which it was assessed how two strategies for reducing dust in hay impacted nutrient contents, horses’ preferences, and horses’ glycemic responses. Another method to refine existing diets is to investigate how ingredients already included in the diet may serve additional purposes. By doing this, diet manufacturers may need to make only small changes to their current formulation for those diets to better serve their consumers. This was the idea behind the second study of this thesis, in which the aim was to determine if a liquid methionine supplement, Alimet® (methionine hydroxy analogue; MHA), as an organic acid could mitigate bacterial contamination from raw ground meat.

7.1. Primary Findings

Previous studies on the changes to hay nutrient contents as a result of soaking or steaming hay have revealed wide variation in their results. Generally speaking, steaming appears to conserve nutrients (Blackman & Moore-Colyer, 1998; Broderick et al., 1993; Earing et al., 2013; Moore-Colyer et al., 2014; Moore-Colyer et al., 2016), while some degree of nutrient losses, including carbohydrates, protein, and minerals have been reported as a result of soaking
hay (Blackman & Moore-Colyer, 1998; Hansen et al., 2016; Longland et al., 2009, 2011, 2014; Mack et al., 2014; Martinson et al., 2012a, 2012b; Moore-Colyer, 1996; Moore-Colyer et al., 2014; Müller et al., 2016; Warr & Petch, 1992; Watts & Sirois, 2003). However, the proportion of these nutrients that are lost appears to be dependent on a number of factors including the fragility of the hay, how readily its nutrients are solubilized, and the temperature and duration of soaking. While the current study was the first to measure nutrient content changes in Canadian timothy-alfalfa hay and the degree of nutrient losses from soaking varied from different hay types measured in other studies, our results were similar to those of others, with notable losses of non-structural carbohydrates and potassium, while these nutrients were conserved in steamed hay. Although the loss of non-structural carbohydrates was expected to elicit a lower glycemic response in horses, no difference was observed in the glycemic response of Standardbred racehorses, which agreed with the results found by Collins (2015). In addition, Standardbred racehorses preferred steamed and dry hay to soaked hay, which better supports the findings of Moore-Colyer & Payne (2012) and Pagan et al (2013), who had smaller sample sizes to work with. Based on these results, it was concluded that since Standardbred racehorses showed preference for steamed hay over soaked and their glycemic response was not influenced by soaking or steaming hay, that steaming hay was the better method to treat hay for performance horses to conserve nutrients to maximize their nutrient intake.

Previous antimicrobial work with MHA has shown it to be bactericidal against *E. coli* in tryptic soy broth at a concentration of 1% (Dibner and Buttin, 2002), and as such it was hypothesized that the minimum inhibitory concentration of Alimet® against *S. Typhimurium* and *L. monocytogenes* in tryptic soy broth would be less than 1%. The results of the experiment agreed with this as it was found that the MIC of Alimet® against *S. Typhimurium* and *L.*
monocytogenes to be 0.3%, and that 0.3% Alimet® was bactericidal against S. Typhimurium and 0.5% Alimet® was bactericidal against L. monocytogenes. Following this, the effectiveness of Alimet® was tested against these pathogens in raw ground meat, intended to mimic a raw meat-based diet in a consumer home by freezing it for 24 hours then thawing it in a refrigerator. Following the 24h freeze and after 24h of refrigeration, it was found that 1.25% Alimet® reduced S. enterica by 20% compared to the control, and reduced L. monocytogenes by 26% compared to the control. Although 1.25% Alimet® did not reduce these pathogens in raw meat to below detection limits, there is still the potential for a higher concentration of Alimet® or Alimet® in combination with other hazard control measures to result in mitigation of pathogenic bacteria.

7.2. Limitations and Future Research

One of the most notable limitations of the study on the effects of soaking and steaming hay is that the results of the glycemic response testing are not applicable to all horses. The horses used in this study were exercise-conditioned Standardbred racehorses and it is known that exercise conditioning improves insulin sensitivity (Pratt et al., 2006), therefore the glycemic responses of these horses should not be assumed to reflect the glycemic responses of other horses, especially those which are known to be insulin resistant. For this reason, future studies investigating the glycemic response of insulin-resistant and non-exercise-conditioned horses are certainly warranted, as the responses of these horses may differ from those in the current study. Despite this limitation, the findings of this study are foundational, and should be used to pave the paths of future research. To better understand how steaming and soaking hay affects nutrient intake by performance horses, future studies should investigate how steaming, as a heat treatment, affects protein availability in hay. As well, based on the findings of this study,
research is also warranted to assess how mineral losses in soaked hay affect electrolyte balance in performance horses.

There were several limitations for the raw meat study presented in this thesis, as this was the first study to investigate an organic acid as an antimicrobial agent for RMBDs. Firstly, there was no published data on contamination levels seen in RMBDs, as previous studies and recalls have only determined the presence or absence of bacteria in these diets. As such, it is unknown if the contamination levels used in this study accurately reflected the bacteria load that would need to be mitigated by antimicrobial agents and hazard control measures. It would serve future researchers and pet food formulators well to determine these levels before proceeding with this type of research, as it may be the case that the contamination levels used in this study were not realistic. Another consideration should be to investigate the effects of diet handling and storage, as the results of the work in this thesis and the PhD dissertation of Hasty (2017) indicate that this may prove to be a useful hazard control measure that is being overlooked. This should be explored in future research, as well as whether these diets are palatable and how acidification of the raw diet affects acid-base balance in the animal consuming it, which in this case it expected to be domestic cats; however, this may be applicable to dogs and other carnivores as well.

7.3. Overall Summary and Implications

In conclusion, it is believed that the findings of both of the studies can be used to improve existing diets for companion animals. In the first study, I determined that steaming hay was the superior method to treat hay for racehorses as it conserved nutrients and was preferred by horses over soaked hay. In the second study, I determined that Alimet® reduces bacterial contamination in raw ground meat, demonstrating another function for this multi-purpose supplement that will make it a valuable addition to RMBDs. Overall, the work presented in this thesis has
demonstrated simple changes that can improve the nutritional integrity, hygienic quality, and palatability of existing companion animal diets.
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