GEL ENCAPSULATION TECHNOLOGY DEVELOPMENT FOR THE PURPOSE OF SAFEGUARDING AN ACTIVE INGREDIENT (HOLY BASIL; OCIMUM SANCTUM) FROM RUMINAL DEGRADATION

by

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ABSTRACT

GEL ENCAPSULATION TECHNOLOGY DEVELOPMENT FOR THE PURPOSE OF SAFEGUARDING AN ACTIVE INGREDIENT (HOLY BASIL; *OCIMUM SANCTUM*) FROM RUMINAL DEGRADATION

James R. Templeman
University of Guelph, 2016

This study aimed to develop an encapsulation technology capable of loading polar and/or apolar active ingredients, in this case ground holy basil leaves, that resists degradation throughout a 48 hour *in vitro* rumen incubation. The gels were formulated by homogenizing a rice-bran wax solution with a sodium alginate solution, creating an oil-in-water emulsion with organogel droplets, followed by a secondary calcium-activated gelation process. The gel’s ruminal stability and the discrete stabilities of the individual constituents were tested *in vitro* via nylon-bag incubations and a gas-fermentation system. The effects of the holy basil form (encapsulated or unencapsulated) were significant in both experiments and the gels demonstrated an average active ingredient retention rate of 81.25% during the nylon-bag analysis. The gas-fermentation trial introduced holy basil’s anti-microbial properties, and GC-MS analysis of the holy basil identified appreciable amounts of eugenol (35.9%) and methyl-eugenol (25.2%), the most influential compounds in terms of its anti-microbial actions.
ACKNOWLEDGEMENTS

First and foremost, I want to express how grateful I am to have had the opportunity to work under the tutelage of Dr. Vern Osborne. Without Vern’s patience, support and overall confidence in me, I assuredly would not be where I am today. I want to offer my gratitude my entire advisory committee of Drs. John Cant, Michael Rogers and Brian McBride, you all proved time and time again how lucky I was to have had such smart, supportive and reliable people to turn to for guidance and insight. I would like to sincerely thank Drs. Amanda Wright and Praveen Saxena for the generous use of their labs and equipment, Dr. Dyanna Brewer at the Dept. of Molecular and Cellular Biology for preforming the GC-MS analysis, Ron Piett at A&L Labs for assisting me with the in vitro nylon-bag trials, and Jay Johnston at RFS Technologies for having me down to his lab in Ottawa to run the in vitro gas-fermentation trials. As well, I want to express my utmost appreciation to the Dairy Farmers of Ontario and the Ontario Center of Excellence for providing the funding that allowed me to, as Vern liked to say, “play in the lab” for the last two years.

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AI</td>
<td>Active ingredient</td>
</tr>
<tr>
<td>CFR</td>
<td>Code of Federal Regulations</td>
</tr>
<tr>
<td>CW</td>
<td>Candelilla wax</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>EHB</td>
<td>Encapsulated holy basil</td>
</tr>
<tr>
<td>FDA</td>
<td>Federal drug administration</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GP</td>
<td>Gas production</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally recognized as safe</td>
</tr>
<tr>
<td>HB</td>
<td>Holy basil</td>
</tr>
<tr>
<td>HBOT</td>
<td>Holy basil on-top</td>
</tr>
<tr>
<td>12-HSA</td>
<td>12-Hydroxystearic acid</td>
</tr>
<tr>
<td>IVDMD</td>
<td>In vitro dry matter disappearance</td>
</tr>
<tr>
<td>IVTD</td>
<td>In vitro true digestibility</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>LMOG</td>
<td>Low molecular-mass organogelator</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NaAlg</td>
<td>Sodium alginate</td>
</tr>
<tr>
<td>NDF</td>
<td>Neutral detergent fibre</td>
</tr>
<tr>
<td>NHB</td>
<td>No holy basil</td>
</tr>
<tr>
<td>OHB</td>
<td>Only holy basil</td>
</tr>
<tr>
<td>OM</td>
<td>Organic matter</td>
</tr>
<tr>
<td>RBW</td>
<td>Rice-bran wax</td>
</tr>
<tr>
<td>SAFiN</td>
<td>Self-assembled fibrillar network</td>
</tr>
<tr>
<td>SIM</td>
<td>Single ion monitoring</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile fatty acid</td>
</tr>
<tr>
<td>Wt%</td>
<td>Weight-percentage</td>
</tr>
</tbody>
</table>
Chapter 1.

**INTRODUCTION**

The emerging consumer interest in value-added, nutrient-enriched products has been a driving force behind the increase in research and development of functional foods. Functional foods are defined as; “(products) similar in appearance to, or may be, a conventional food, consumed as part of a usual diet, which is demonstrated to have physiological benefits and/or to reduce the risk of chronic disease beyond basic nutritional functions” (Agriculture and Agri-Food Canada, 2015). Functional foods are rapidly gaining popularity and considering their widespread consumption, availability, and versatility, milk and milk products represent ideal candidates for functional food applications.

Nutritionally fortified milk products have the potential to benefit a wide range of consumers by promoting healthier food choices in school cafeterias, grocery stores, and assisted living communities. Fortifying milk by supplementing the diet of the cow allows for the active ingredients (AIs) to be naturally passed into the milk via the cow’s bloodstream. However, pregastric fermentation in ruminant animals poses a major challenge when attempting to deliver ingested AIs into the blood, particularly for AIs that are sensitive to the destructive elements within the cow’s rumen, such as physical digestion, microbial fermentation, pH, and temperature fluctuations. One approach to counter this roadblock entails employing encapsulation technology to safeguard AIs from rumen degradation in hopes of increasing the value of the milk by naturally fortifying it with functional ingredients.

As a gelation agent, sodium alginate (NaAlg) is versatile and innocuous, and the
stable polymeric gel matrix it forms can safeguard encapsulated AIs from external stressors (Funami et al., 2009; Lupo et al., 2014). Extracted from the cell walls of brown seaweed (Macrocystis pyrifera), NaAlg is considered an ideal candidate for the encapsulation and delivery of hydrophilic AIs or volatile bioactives such as plant polyphenols (Florián-Algarín & Acevedo, 2010; Lupo et al., 2014).

Sodium alginate gelation is activated upon interaction with divalent cations such as $\text{Ca}^{2+}$, and this activation can occur in one of two ways: externally, at a neutral pH, or internally, via acidification of the medium (Poncelet et al., 1995; Funami et al., 2009; Lupo et al., 2014). External gelation incorporates a water-soluble form of calcium (eg. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) that gels the NaAlg immediately upon interaction, however, with an insoluble calcium salt (eg. $\text{CaCO}_3$), NaAlg gelation only occurs following acidification of the NaAlg solution. With a drop in pH, free $\text{Ca}^{2+}$ is liberating from the insoluble salt, inducing NaAlg gelation (Funami et al., 2009; Lupo et al., 2014). This study utilized both stages of calcium-activated gelation: rapid and immediate (external) gelation via $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, as well as a continuous (internal) gelation via gradual liberation of $\text{Ca}^{2+}$ from insoluble $\text{CaCO}_3$ once the gel comes in contact with rumen fluid, causing a drop in pH, during the in vitro incubations.

By incorporating a wax-in-oil (organogel) complex into the gel product, the potential exists to encapsulate hydrophobic, lipid-soluble AIs within the gel-matrix, in addition to hydrophilic, water-soluble AIs. Organogels are formed via low molecular-mass organogelators (LMOGs) that are dissolved in an organic liquid, creating a solution. Once the solution cools, a sol-to-gel transformation occurs, and the organogel is stabilized via the formation of a self-assembled fibrillar network (SAFiN) that traps the
lipophilic AI and the organic solvent (Abdallah & Weiss, 2000). Rice-bran wax (RBW) is a rumen-stable, non-toxic, economical and readily available organogelator that displays high crystallinity, allowing for it to gel organic liquids at low concentrations (Dassanayake et al., 2009).

Holy basil (HB; Ocimum sanctum), also known as Tulsi, is an aromatic plant widely cultivated across South Asia. It is believed to be religiously sacred and HB’s secondary metabolites are known to endow it with a number of medicinal, health-promoting properties, including anti-stress (Gupta et al., 2007; Singh et al., 2012), anti-oxidative (Vrinda & Devi, 2001), anti-ulcerogenic (Dharmani et al., 2004), and anti-microbial actions (Gupta et al., 2002; Viyoch et al., 2006). Holy basil is regarded as being an economical and widely available preventative measure for potential deficiencies, and treatment for various ailments (Singh et al., 2012).

The two major experiments conducted for this project were aimed at the development of gel encapsulation technology designed to safeguard an AI (HB; Ocimum sanctum) from ruminal degradation. Determining the in vitro ruminal degradability and fermentative gas production rates of the encapsulation technology will allow for an estimation of the rumen bypass potential, and thereby determine if a RBW and NaAlg formulation is suitable to enhance transfer efficiency of HB from the rumen to the intestine for absorption into blood. This pilot project took aim at laying the groundwork for natural milk fortification using gel encapsulation technology as a target-specific delivery system for mammary gland nutrient deposition.
References (1.1)


Chapter 2.

LITERATURE REVIEW

Enriched Milk Products (2.1)

The presence of value-added products, such as functional foods, in North America’s health industry has grown substantially in recent years, with products such as omega-3 enriched eggs rapidly gaining consumer acceptance. Milk represents an ideal candidate for functional food applications due to its widespread consumption and past success in terms of novel product development. For instance, the University of Guelph’s Department of Animal Biosciences, led by Dr. Brian McBride, formulated a docosahexanoic acid (DHA) rich feed supplement that, when fed to dairy cows, resulted in higher levels of DHA in their milk (Wright et al., 1999). This research group teamed with Neilson Dairy to successfully develop and brand Dairy Oh! Milk™, a DHA-enriched milk product. This DHA (omega-3 fatty acid) enrichment helps maintain neural function and physical brain development while improving visual acuity and cognitive function, especially in infants and young children (Horrocks & Yea, 1999).

Fortification (2.1.1)

Fortified milk has the potential to promote healthier food choices for people of all ages by providing nutrient-enriched product options in grocery stores, school cafeterias, and assisted living communities. Fortifying milk by supplementing the diet of the cow itself allows for active ingredients to be naturally incorporated into the milk via the cow’s metabolic machinery. Diet-level fortification has the potential to appeal to consumers who may be comparing this natural, cow-side approach to the alternative, post-processing methods of nutrient-enrichment. Pregastric fermentation in ruminants remains a barrier to
achieving high loading efficiency when attempting natural enrichment with more degradable, orally consumed active ingredients. In an attempt to counter the issue of ruminal degradation and improve active ingredient transfer efficiency, encapsulation techniques must be employed.

Organogels (2.2)

Hughes et al. (2009) define an organogel as; “an organic liquid entrapped within a thermo-reversible, three dimensional gel network”. Organogels result from the dissolution and crystallization of low concentrations (0.5 – 2.0 weight-percentage [wt%]) of an organogelator (eg. LMOG) into a hydrophobic organic liquid (Hughes et al., 2009). The liquid is heated to a temperature above the melting point of the organogelator, creating a solution. Once in the solution state, it is cooled to a temperature suitable for a solution-to-gel transition. At this stage, the organogelator molecules form a SAFiN that is stabilized through non-covalent interactions to immobilize the organic solvent (Abdallah & Weiss, 2000).

Organogel Utilization (2.2.1)

Organogelation has been industrially utilized as far back as the 1970’s for mechanical lubrication, crude oil spill recovery, and in cosmetics (Abdallah & Weiss, 2000). Their use in food and pharmaceutical industries has picked up noticeably in recent years largely due to organogels’ adaptability, gel matrix stability at low organogelator concentrations, and ability to act as controlled-release delivery systems for lipophilic bioactive ingredients (Abdallah & Weiss, 2000; Hughes et al., 2009). As well, much interest in organogelation lies in the advent of mono- and poly-unsaturated fatty acids
potentially replacing hardstock *trans*- and saturated fats when structuring edible oils in food products, while still maintaining the texture, taste, and mouth-feel of the food (Dassanayake *et al.*, 2009; Hughes *et al.*, 2009; Dassanayake *et al.*, 2012). The fat crystal networks formed by traditional hardstock fats provides many foods with their desired sensory properties; however, there is significant evidence relating elevated intake of *trans*- and saturated fats to an increased risk of developing cardiovascular diseases (Rogers, 2009).

**Organogel Characteristics (2.2.2)**

Organogels can form in a highly effectively manner with a very low LMOG concentration. While in most cases a 2 wt% organogelator concentration is utilized for gelation, organogelator concentrations as low as 0.5 wt% have successfully formed self-standing organogels (Abdallah & Weiss, 2000; Dassanayake *et al.*, 2009; Hughes *et al.*, 2009). As the solution-to-gel transition is occurring between the hydrophobic solvent and the LMOG, lipophilic bioactive substances can be trapped within the SAFiN along with the organic liquid (Iwanaga *et al.*, 2010). The organogel can act as a controlled-release carrier for bioactive substances into a biological system, where the release rate of the lipophilic compound depends on the LMOG concentration in the gel formulation as well as the environmental conditions (eg. pH, temperature, etc.) of the biological system (Iwanaga *et al.*, 2010). Following consumption by an animal, the organogel degrades, causing the oil and bioactive substance to be gradually released into the gastrointestinal tract (Iwanaga *et al.*, 2010). The solution-to-gel transition is thermally reversible; thus, the temperature of the organogel can be repeatedly raised above or cooled below the gelation temperature (Abdallah & Weiss, 2000).
Organogelators (2.3)

Three organogelators will be considered for the dispersed phase of a final emulsion: 12-hydroxystearic acid (12-HSA), candelilla wax (CW), and rice-bran wax (RBW). These gelators were chosen based on the criteria that they are non-toxic, economical in comparison to alternative organogelators such as ceramides, readily available for purchase in North America, possess existing literature detailing their use in the formation of organogels, and are capable of creating a stable gel-matrix at low concentrations. Table 2-1 outlines the chemical characteristics and Federal Drug Administration (FDA) affirmations for each of 12-HSA, CW, and RBW.

Table 2-1. Chemical characteristics and FDA affirmation for the three organogelators (RBW, CW & 12-HSA) selected for this study.

<table>
<thead>
<tr>
<th></th>
<th>Rice-bran Wax</th>
<th>Candelilla Wax</th>
<th>12-Hydroxystearic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting point (°C)</td>
<td>77 - 82</td>
<td>68.5 – 72.5</td>
<td>72 - 78</td>
</tr>
<tr>
<td>Iodine value (g Iodine/100g)</td>
<td>≤ 20</td>
<td>13-23</td>
<td>&lt; 4</td>
</tr>
<tr>
<td>Acid value (mg KOH/g)</td>
<td>≤ 13</td>
<td>12 - 22</td>
<td>&gt; 175</td>
</tr>
<tr>
<td>Saponification value (mg KOH/g)</td>
<td>75 - 120</td>
<td>43 - 65</td>
<td>&gt; 175</td>
</tr>
</tbody>
</table>

*a Koster Keuken sample datasheets
*b US Federal Drug Administration (FDA) Code of Federal Regulations (CRF) Title 21

In 2009, Dassanayake et al. found that rice-bran wax could form a stable organogel with olive oil, salad oil (50:50 canola:soybean oil) and liquid paraffin at a concentration as low as 0.5 wt%. The same study tested gelation capabilities of candelilla wax and found that a minimum concentration of 2 wt% was required for gel formation to occur (Dassanayake et al., 2009). Rogers et al. (2008) demonstrated the ability of 12-HSA to form weak, but relatively self-standing gels at a concentration as low as 0.5 wt%. However at commonly utilized concentrations of 2 wt%, the gel became discernably heterogeneous and became opaque. It was stated though, that at a concentration as low as 0.3 wt%, a solution-to-gel transformation did initiate, but the
solution only began to thicken and did not fully transition to a self-standing gel (Rogers et al., 2008). It should be noted that the polarity of the organic solvent, as well as the cooling rate and storage temperature influences the minimum gelation concentration of these organogelators (Dassanayake et al., 2011).

12-Hydroxystearic Acid (HSA; 12-Hydroxyoctadecanoic Acid) (2.3.1)

12-hydroxystearic acid is an 18-carbon saturated fatty acid formed as a result of a substitution of a hydroxyl group at the 12\textsuperscript{th} carbon of stearic acid. Ricinoleic (90%), oleic, and stearic acids comprise castor oil, and upon the full hydrogenation of castor oil, 12-HSA is formed. The hydrogenated oil is saponified with KOH and acidified with a mineral acid to create a commercially available, technical-grade form of 12-HSA. This crude preparation is approximately 85% 12-HSA and 15% stearic acid. This technical-grade, optically active D-12-HSA, as well as its racemic counterpart (\textit{DL}-12-HSA), are both able to gel organic oils (Marangoni, 2012). 12-hydroxystearic acid has been utilized for decades in the manufacturing of greases for lubrication. Additionally, because of its ability to firmly gel oils at low concentrations, 12-HSA has also been used as a method to safely dispose of waste oils from the food and automobile industry (Marangoni, 2012).

12-HSA Chemical Composition (2.3.1.1)

The hydroxyl groups present in 12-HSA are largely responsible for its efficiency as an organogelator (Toro-Vazquez \textit{et al.}, 2013). The two hydroxyl groups of 12-HSA create a more polar molecule with an increased capacity for hydrogen bonding when compared to stearic acid. The structural change, caused by the addition of the hydroxyl group at carbon-position 12, limits 12-HSA hydrocarbon interactions, but the increased
hydrogen bonding overcomes that loss in structural strength (Toro-Vazquez et al., 2013). When compared to stearic acid, half as much 12-HSA was needed to successfully gel edible organic oil, supporting the notion that derivatized fatty acids are more efficient organogelators than fatty acids (Dassanayake et al., 2011).

**12-HSA FDA Affirmation (2.3.1.2)**

12-hydroxystearic acid is considered an indirect food additive in accordance with FDA’s Code of Federal Regulations, Title 21, Chapter 1, Subpart D, Part 178, Section 178.3570. 12-hydroxystearic acid is accepted as a lubricant with incidental food contact (with no restrictions) that can be safely used on machinery that produces, packages, processes, transports, stores and prepares food (FDA, 21 CFR 178,3579, 2015). Since 12-HSA is derived from castor oil, a known laxative, the compound’s potentially disruptive effects if ingested in large amounts by humans prevent it from gaining consideration as an acceptable food additive (Rogers, 2009).

**Euphorbia Cerifera (Candelilla) Wax (CW) (2.3.2)**

Candelilla wax is derived from the leaves of *Euphorbia cerifera* (or *Euphorbia antisypilitica*), a small shrub that is native to northern Mexico and the southwestern United States (Toro-Vazquez et al., 2007; Rocha et al., 2013; Rogers et al., 2014). Though well studied as a food-grade organogelator, CW is largely used as a glaze or chewing gum binder in the food industry, as a constituent in lip balms or lotions in the cosmetic industry, and as a varnishing agent in the paint industry. The long-term stability of CW and its textural properties make it desirable candidate for usage in the food industry. The stability of CW was demonstrated by Toro-Vazquez et al. (2007) as the
group observed no visible phase separation within a 3 wt % CW organogel over a 3 month period of room-temperature storage.

**CW Chemical Composition (2.3.2.1)**

Structurally, CW is composed largely of 28-33 carbon-atom n-alkanes (44-45%), with lesser constituents including alcohols of penta-cyclic triterpenoids, 18-34 carbon-atom aliphatic acids, 24-34 carbon-atom aliphatic alcohols, and esters of alcohols of penta-cyclic triterpenoids. At 75.9%, hentriacontane \((\text{CH}_3(\text{CH}_2)_{29}\text{CH}_3)\) is the most prominent of the n-alkanes in CW (Toro-Vazquez et al., 2013; Rogers et al., 2014). Much of the functionality of CW as an organogelator is credited to the congregation and interactions of the n-alkanes (Rogers et al., 2014). Candelilla wax exhibits a platelet-style crystallization network during the solution-to-gel transformation. This crystal network imparts CW organogels with respectable rheological properties due to the shear number of crystals formed and the high degree of crystal-on-crystal interaction (Toro-Vazquez et al., 2013). Toro-Vazquez et al. (2007) found that a CW concentration of at least 2 wt% was required to form a stable, opaque, self-standing organogel without any dependence on the storage temperature. The same experiment demonstrated that when storage temperature was reduced down to 5°C, a 0.5 wt% CW concentration began to thicken the sunflower oil solution, and a 1 wt% concentration produced a translucent gel, though one that still flowed when the test tube was inverted (Toro-Vazquez et al., 2007).

**CW FDA Affirmation (2.3.2.2)**

Candelilla wax has been affirmed as generally recognized as safe (GRAS) as a direct human food ingredient in accordance with FDA’s Code of Federal Regulations,
Title 21, Chapter 1, Subpart B, Part 184, Section 184.1976. Aside from current good manufacturing practice conditions, no restrictions have been placed on CW for use as a food ingredient (FDA, 21 CFR 184.1976, 2015).

Oryza Sativa (Rice) Bran Wax (RBW) (2.3.3)

Rice-bran wax, in its highest purity (>99%), consists of a natural, pale yellow, odorless wax that is derived from rice bran during the extraction of rice-bran oil from the bran itself (Vali et al., 2005; Dassanayake et al., 2009). Refining rice-bran oil from rice-bran (a by-product of rice milling) typically occurs in stages: dewaxing, degumming, deacidification, bleaching, and deodorization. The wax portion is obtained during the dewaxing stage, although this initial wax is a crude, impure wax with a dark brown resinous matter throughout. That crude wax undergoes a solvent-defatting process to produce a dry, powdered wax. A bleaching process follows defatting to ultimately yield highly pure, off yellow, odorless wax crystals that are finally saponified with KOH (Vali et al., 2005). Rice-bran wax is largely used as a wax coating for fruits and vegetables, as well as in the pharmaceutical, cosmetic, and leather industries (Dassanayake et al., 2009).

RBW Chemical Composition (2.3.3.1)

The chemical composition of RBW varies based on the method of extraction and purification, though it is largely composed of long carbon chain fatty acids (C\textsubscript{16} – C\textsubscript{32}) esterified to fatty alcohols (C\textsubscript{24} – C\textsubscript{38}) with the most prevalent fatty acid and fatty alcohol being lignoceric acid (C\textsubscript{24}) and tricontanol (C\textsubscript{30}), respectively (Vali et al., 2005; Dassanayake et al., 2011). With a relatively high melting point, in the range of 77-82°C, RBW displays high crystallinity at ambient temperatures when compared to typical plant based waxes such as carnauba or candelilla wax (Dassanayake et al., 2009). The long,
thin, needle-like shape of the RBW crystals allow for a much finer dispersion in liquid oils versus the crystals of carnauba and candelilla wax. Very likely due to the high rate of crystal dispersion, the minimum concentration needed for RBW to form a stable organogel in olive oil was just 0.5 wt%, compared to 4 and 2 wt% for carnauba and candelilla waxes, respectively (Dassanayake et al., 2009).

**RBW FDA Affirmation (2.3.3.2)**

Rice-bran wax is considered a food additive that has been permitted for direct addition to food for human consumption in accordance with FDA’s Code of Federal Regulations, Title 21, Chapter 1, Subpart B, Part 172, Section 172.890. It is currently used, or intended for use, as a coating for candy, fruits and vegetables, as well in the formulation of chewing gum (FDA, 21 CFR 172.890, 2015).

**Sodium Alginate (2.4)**

Sodium alginate (NaAlg) is obtained via a two-stage extraction process that begins with an alginic acid extraction from the cell walls of brown seaweed (algae) species. NaAlg itself is then extracted from the sodium salts of alginic acid (Florán-Algarín & Acevedo, 2010). Alginate’s chemical composition can differ greatly between seaweed species, and the specific parts of a seaweed species that have been used in the extraction also alter the alginate composition (Fu et al., 2011). Alginate’s simplicity and non-toxicity have led food and pharmaceutical industries to readily use NaAlg as a gelling, thickening, and immobilization agent (Florán-Algarín & Acevedo, 2010). However, in recent years the focus around NaAlg in the food industry has been turned to its use as an additive to foods and beverages for the purpose of reducing postprandial appetite, food intake, and glycaemia. The soluble, viscous gel formed from alginate has
been shown to help induce satiety and control glucose homeostasis by reducing glucose and insulin concentrations following a meal (Khoury et al., 2014). As a gelation agent, NaAlg is versatile and innocuous, and the stable polymeric gel matrix it forms can safeguard encapsulated AIs from external stressors (Funami et al., 2009; Lupo et al., 2014). Sodium alginate is considered an ideal candidate for the encapsulation and delivery of hydrophilic active ingredients or volatile bioactives such as probiotic bacteria (Florián-Algarín & Acevedo, 2010; Lupo et al., 2014).

**Mannuronic-Guluronic (MG) Blockiness (2.4.1)**

Alginate, a natural polysaccharide, is a linear, unbranched copolymer that is molecularly composed of blocks of uronic acid residues (Leng et al., 2010; Khoury et al., 2014). These monomers are made up of (1-4)-linked β-D-mannuronic acid and α-L-guluronic acid residue segments that make up M-, G-, or MG-rich block structures (Khoury et al., 2014). These three block structure types are shown in Figure 2-1. The ratios of these residues (M/G), as well as the distribution of these monomers affect the capability of NaAlg to form a gel when exposed to divalent cations such as Ca$^{2+}$ (Funami et al., 2009; Florián-Algarín & Acevedo, 2010; Leng et al., 2010). However, aside from the uronic residue ratio and distribution, the concentration of NaAlg in solution also influences the viscosity, elasticity, and strength of the formed gel (Khoury et al., 2014).

Calcium can only bind to GG- and MG-block structures and, by way of “egg-box” dimerization, induce alginate gelation (Leng et al., 2010). Due to this selective Ca$^{2+}$ binding, alginates rich in G-block residues will form hard and inelastic gels, while those rich in M-block residues will form soft and elastic gels (Funami et al., 2009). MM-block
structures act as the solvating portion of the alginate molecule, allowing for the water absorption and swelling that characterises elastic gels (Leng et al., 2010).

Figure 2-1. The monomeric blocks that make-up alginate; consisting of homogeneous GG- and MM-residues, as well as heterogeneous MG-blocks (Adapted from Fu et al., 2011).

Funami et al. (2009) describe a ratio of Ca$^{2+}$ to each G-residue unit ($R_{[Ca][G]}$) that must be kept within minimum (0.25) and maximum (0.55) critical values in order for alginate gel formation in the presence of Ca$^{2+}$ to occur. The NaAlg used for this study was prepared from an American strain of the seaweed species *Macrocystis pyrifera* which, according to literature (Haug & Larsen, 1962; Morris, Rees & Thom, 1980; McHugh, 1987; Fu et al., 2011), has a mannuronic:guluronic acid ratio of 1.56:1 (0.39 [G] to 0.61[M] fractional composition).
Calcium Salt Alginate Gelation Activation (2.4.2)

The gelation process for alginate is activated by a cationic interaction with Ca$^{2+}$ that can essentially occur in one of two ways: externally, at a neutral pH, or internally, via acidification of the medium (Poncelet et al., 1995; Funami et al., 2009; Lupo et al., 2014). External gelation, the more widely utilized encapsulation technique, involves administration of a water-soluble Ca$^{2+}$ source (e.g., CaCl$_2$$\cdot$$2$H$_2$O) that immediately activates gelation via cationic interaction when added to a NaAlg solution. Alternatively, internal gelation occurs when a drop in pH causes the release of Ca$^{2+}$ from an insoluble Ca$^{2+}$ salt (e.g., CaCO$_3$) already present in the NaAlg medium. Once liberated from its inactive form, the Ca$^{2+}$ is free to interact with the NaAlg (Funami et al., 2009; Lupo et al., 2014). Lupo et al. (2014) determined that for stable gelation of a 2 wt% NaAlg solution, a minimum of $1.13 \times 10^{-4}$ mol of Ca$^{2+}$ was required per gram of NaAlg (e.g., 0.018 g CaCO$_3$ or 0.01997 g CaCl$_2$$\cdot$$2$H$_2$O per g NaAlg). The pH of the rumen for a healthy cow will fluctuate throughout the day, dropping down as low as a pH of 5.5, though the ideal pH range for ruminal fermentation is generally from 6.0 to 6.4 (Mutsvangwa & Wright, 2003): a range utilized by the in vitro systems during the gas-style and nylon-bag rumination trials. Ca$^{2+}$ release from an insoluble salt can occur with a pH from 7.5 to 6.5, which then initiates the gel formation between Ca$^{2+}$ and alginate (Reis et al., 2006).

Ca$^{2+}$ Induced Gel Formation via the “Egg-Box” Gelation Model (2.4.3)

The cationically induced gelation of NaAlg, termed the “egg-box” gelation model (Figure 2-2), occurs as Ca$^{2+}$ ions (the “egg”) interact with oxygen atoms in cavities (the “box”) formed by the alignment and pairing of guluronic acid monomers (Fu et al., 2011).
Figure 2. Depiction of the “egg-box” structure and dimerization between $\text{Ca}^{2+}$ (unfilled circles) and the oxygen atoms (filled circles) within G-blocks (zigzags) (Adapted from Fu et al., 2011).

Alginate gel formation relies on three distinct steps (Figure 2-3) that sequentially occur with the consistent addition of free cations (e.g., $\text{Ca}^{2+}$): 1) monocomplexation, 2) “egg-box” dimerization, and 3) laterally associated multimerization (Funami et al., 2009; Fu et al., 2011). When $R_{[\text{Ca}]/[\text{G}]} < 0.25$ is reached (minimal critical boundary), the free $\text{Ca}^{2+}$ interacts with a single G-monomer forming a more compact mono-complex. The next step consists of a $0.25 < R_{[\text{Ca}]/[\text{G}]} < 0.55$ that induces “egg-box” dimerization via pairwise linking of two monocomplexes. Once an $R_{[\text{Ca}]/[\text{G}]} > 0.55$ is reached, lateral associations between the egg-box dimers cause the formation of intracluster associated multimers (Funami et al., 2009; Fu et al., 2011).
Figure 2-3. Illustration of three-stage Ca\(^{2+}\) binding induced alginate gelation with relative Ca\(^{2+}\) to G-residue ratios ($R_{[Ca]/[G]}$) required for each step to occur. G-blocks, M-blocks and Ca\(^{2+}\) described with zigzag lines, straight lines and dots, respectively (Adapted from Funami et al., 2009).

**NaAlg FDA Affirmation (2.4.4)**

Sodium alginate has been affirmed as generally recognized as safe (GRAS) as a direct human food ingredient in accordance with FDA’s Code of Federal Regulations, Title 21, Chapter 1, Subpart B, Part 184, Section 184.1724. Sodium alginate has been permitted for use as a food ingredient in the processing of condiments, hard candies, gelatins, and fruit juice, as well as for use as an emulsifying, stabilizing, or firming agent in all other food categories (FDA, 21 CFR 184.1724, 2015).
Holy Basil (HB; *Ocimum sanctum* Linn; Tulsi) (2.5)

Holy basil (HB; *Ocimum sanctum* L.), also known as Tulsi, is an aromatic, perennial plant from the family *Lamiaceae* that has been widely cultivated across South Asia for over 3000 years (Gupta *et al*., 2002; Satyanarayana & Sen, 2009). Three varieties of Tulsi exist: 1) Rama/Shri/Lakshmi/Light Tulsi, 2) Krishna/Shyama/Dark Tulsi, and 3) Vana/Wild Tulsi (Satyanarayana & Sen, 2009; Singh *et al*., 2012). Each type varies slightly in colour, aroma and chemical composition, however environmental growth factors (eg. edaphic and geographic) can also cause chemical property variation within the same strain of HB (Mondal *et al*., 2009). As a medicinal constituent, HB is regarded as being inexpensive and widely available (Juntachote & Berghofer, 2005). Recent advances in plant propagation techniques have allowed for the growth of HB in Europe, Australia, and North America. (Singh *et al*., 2012; Pandey, *et al*., 2014).

Aside from its medicinal uses, HB is commonly utilized as an aromatic agent in cosmetics, as well as in the food industry as a flavouring agent in sauces, soups, salad dressings and sausages. Holy basil leaves are also often dried and steeped in boiling water to make a fragrant, herbal tea (Satyanarayana & Sen, 2009; Pandey *et al*., 2014). Holy basil and its’ essential oil have been affirmed as substances that are generally recognized as safe (GRAS) for human consumption in accordance with FDA’s Code of Federal Regulations, Title 21, Chapter 1, Subpart B, Part 182, Section 182.20 (FDA, 21 CFR 182.20, 2015).
Cultural Importance of Holy Basil (2.5.1)

Both Rama and Shyama Tulsi are revered as being religiously sacred and worshipped in Hinduism to such an extent that a Hindu home without a Tulsi plant is considered to be an incomplete home (Singh et al., 2012). In Sanskrit, “Tulsi” translates to “the incomparable one”, and scriptures of Hinduism dating back to the Vedic times (c. 1500 – c. 500 BCE) speak of HB’s extensive use in treating various ailments such as asthma and insomnia (Satyanarayana & Sen, 2009; Singh et al., 2012). Charaka Samhita, one the two foundational texts based on the central teaching of Ayurveda medicine, written around 1,000 BCE, makes mention of HB as a traditional curative medicine (Mondal et al., 2009; Pattanayak et al., 2010).

Phytochemical Composition of Holy Basil (2.5.2)

The chemical make-up of HB can vary greatly between strains, or even within the same strain depending on the conditions of the environment (eg. planting season, time of harvest, geography, soil composition, etc.) in which it was grown (Mondal et al., 2009; Pattanayak et al., 2010; Sims et al., 2014). The oil derived from HB leaves, referred to as “volatile” or “essential” oil contains, a number of biologically active constituents including, but not limited to, eugenol, methyl-eugenol, carvacrol, camphor, linalool, naphthalene, caryophyllene, ursolic acid, ascorbic acid, and rosmarinic acid (Hakkim et al., 2007; Pattanayak et al., 2010; Singh et al., 2012). Two water-soluble flavonoids, orientin and vicenin, have also been isolated in HB’s volatile oil (Vrinda & Devi, 2001). However, the number and proportion of these constituents present in a given sample of essential oil varies greatly depending on the extract technique, HB strain, and propagation...
conditions (Pattanayak et al., 2010; Sims et al., 2014). The oil extracted from HB seeds is referred to as “fixed” or “non-volatile” oil and is mainly comprised of five fatty acids: linoleic, linolenic, oleic, palmitic, and stearic acids. Proportions of each of these fatty acids vary depending on plant strain or growing conditions (Singh et al., 2007).

**Health-Promoting Properties of Holy Basil (2.5.3)**

The essential oils and abundance of secondary metabolites in HB endow it with a number of medicinal, health-promoting properties, including adaptogenic/anti-stress (Bhargava & Singh, 1981; Gupta et al., 2007), radiation-protective (Vrinda & Devi, 2001), anti-oxidative (Juntachote & Berghofer, 2005; Juntachote et al., 2007), anti-ulcerogenic (Dharmani et al., 2004), and anti-microbial actions (Viyoch et al., 2006).

**Adaptogenic Actions of Holy Basil (2.5.3.1)**

An ethanolic HB extract (100 mg/kg body weight dosage) effectively normalized hyperglycemia, plasma creatine kinase levels and adrenal gland weights in rats exposed to acute and chronic unpredictable stressors (Gupta et al., 2007). As well, methanolic HB extract (400 mg/kg body weight dosage) has been shown to improve the survival time, a model for physical endurance, of swimming mice and prevent ulceration in rats exposed to stressful environmental changes (Bhargava & Singh, 1981).

**Radiation-Protective Actions of Holy Basil (2.5.3.2)**

Orientin and vicenin, two water-soluble flavonoids that have been isolated and identified in an aqueous HB extract, are shown to effectively radiation-protect culture-based human blood lymphocytes against chemotherapy level doses of radiation (Vrinda
& Devi, 2001). Non-toxic concentrations of both these flavonoids effectively protected cultured human peripheral lymphocytes against both apoptosis and chromosomal irregularities induced by $\gamma$-radiation. As well, orientin and vicenin have been shown to radiation-protect mouse bone marrow chromosomes from aberrations and increase the survival rate of mice exposed to lethal doses of $\gamma$-radiation. The radiation-protective abilities of these two flavonoids are generally attributed to their anti-oxidant actions (Vrinda & Devi, 2001).

**Anti-Oxidative Actions of Holy Basil (2.5.3.3)**

A number of studies have demonstrated the anti-oxidative properties of HB and introduced the prospect of replacing potentially carcinogenic synthetic antioxidants (eg. butylated hydroxyanisole; BHA, or buylated hydroxytoluene; BHT) with natural plant extract-based antioxidants for the purpose of food preservation (Juntachote & Berghofer, 2005; Hakkim *et al.*, 2007).

Using a number of antioxidant activity models (eg. superoxide anion scavenging, DPPH-scavenging, Fe$^{2+}$ chelating ability, etc.), HB ethanolic extract was shown to be highly effective at both scavenging superoxide anion free radicals and chelating ferrous Fe$^{2+}$ ions in a concentration-dependent manner that plateaued at a 1.0 mg/ml concentration (Juntachote & Berghofer, 2005). The HB extract efficiently inhibited lipoxygenase, successfully repressing a pathway that eventually leads to oxidative degradation of polyunsaturated fatty acids causing lipid rancidity in stored foods. Compounds that can impede this pathway have also been shown to inhibit tumour growth in animal models (Juntachote & Berghofer, 2005).
When studied as a natural food preservative, HB (as both a dried powder and ethanolic extract) successfully protected cooked ground pork from lipid oxidation for 14 days when stored at 5°C. Interestingly, ground and dried HB leaves at a 0.35% concentration proved more effective in impeding lipid oxidation than a range of concentrations of ethanolic HB extract (Juntachote et al., 2007).

**Anti-Ulcerogenic Actions of Holy Basil (2.5.3.4)**

A study in 2004 by Dharmani et al. demonstrated the anti-ulcerogenic and ulcer-healing actions of HB. Rats and guinea pigs were exposed to various ulcerogens (eg. cold restraint, aspirin, alcohol, histamine, etc.) in order to induce both gastric and duodenal ulcers. Chronic ulceration was also induced using acetic acid so as to study ulcer-healing activity. An ethanolic HB extract was orally administered and its effects were compared to omeprazole, a standard anti-ulcer drug. At a dose of 100 mg/kg body weight, HB extract significantly decreased the instances of ulceration in all models and HB’s anti-ulcerogenic actions effectively out-performed omeprazole in the case of alcohol-induced ulcers. As well, the HB extract significantly healed ulcers at all three time-points measured, and fully healed ulcers after 20 days, doing so with a higher protection percentage than omeprazole (Dharmani et al., 2004).

**Anti-Microbial Actions of Holy Basil (2.5.3.5)**

A number of studies have demonstrated the anti-microbial/bacterial activity of HB against both Gram-positive (eg. *Staphylococcus aureus*) and Gram-negative (eg. *Escherichia coli*) disease-causing bacteria (Gupta et al., 2002; Viyoch et al., 2006; Chandrasekaran et al., 2012). Both volatile (leaves) and fixed (seeds) oil extracts of HB
have been shown to exhibit strong anti-bacterial properties, inhibiting the growth of a number of bacterial strains including *S. aureus*, *E. coli*, *Bacillus pumilus*, *Propionibacterium acnes* and *Pseudomonos aeruginosa* (Prakash & Gupta, 2005; Viyoch *et al.*, 2006; Pattanayak, *et al.*, 2010). One particular study developed an alternative drug delivery system based around treating fabrics (woven t-shirts) with medicinal herbal extracts as a remedy for bacterially related diseases. The HB-treated fabric proved highly effective, exhibiting zones of inhibition (diameter in mm) of 30 and 21, and bacterial reduction rates (compared to an untreated control) of 94 and 78%, for *S. aureus* and *E. coli*, respectively (Chandrasekaran *et al*., 2012). Singh *et al.* (2013) compared the antimicrobial activity (zone of inhibition) of methanolic and aqueous HB extracts (root and leaf) against *E. coli*, *S. aureus*, and *Proteus mirabilis*. The methanolic extract outperformed the aqueous extract in all cases, exhibiting larger zones of inhibition against each bacterial strain at every concentration used, with the exception of *E. coli*, which showed resistance to both HB leaf extracts.

Eugenol and methyl-eugenol, two of the major constituents that make up HB’s essential oil, are believed to be the most influential in terms of HB’s anti-microbial effect (Viyoch *et al.*, 2006). It has been reported that the hydroxyl groups attached to those compounds’ respective aromatic rings provide them with their highly effective antimicrobial abilities (Farag *et al*., 2004; Soltan *et al*., 2007). As stated earlier, many factors can cause variation in the chemical composition of the oil extracted from HB leaves, however, in most cases, eugenol and methy-eugenol make-up large portions of the volatile oil. Eugenol has been identified in HB extract in a variety of quantities including 40% (Prakash & Gupta, 2005), 41.5% (Viyoch *et al.*, 2006), 59.4% (Trevisan *et al.*, 2005).
2006), and 71% (Gupta et al., 2002). With a similarly wide range of variation, methyl-
eugenol has been identified in quantities including 11.8% (Viyoch et al., 2006), 20% (Gupta et al., 2002), and upwards of 78.4% (Kothari et al., 2004).

Eugenol itself has been used as a natural agent to modify the microbial
environment and fermentative process within the rumen. However, in vitro studies have
indicated that the effects of eugenol on the ruminal microbial environment can be
variable depending on the diet fed, dosage used, and in vitro technique implemented
(Benchaar et al., 2012). Supplementing both high- and low-concentrate TMRs for dairy
cows with eugenol reduced the acetate:propionate ratio as well as protozoa counts in
ruminal fluid (Benchaar et al., 2012). For the low-concentrate diet, eugenol
supplementation reduced in sacco organic matter (OM) degradation of grass hay, soybean
meal and corn grain while lowering Selenomonas ruminantium counts in the liquid phase
of rumen contents, Ruminococcus spp counts in the solid phase, and Prevotella spp. counts
in both phases. Eugenol addition also reduced Selenomonas ruminantium counts in both
phases of rumen contents on the high-concentrate diet (Benchaar et al., 2012).

Soltan et al. (2011) studied the in vitro capability of carvacrol and eugenol to
modify the rumen microbial fermentation and found that both additives, at varying
concentrations, significantly reduced the ruminal GP (caused by microbial fermentation),
as well as the OM degradation and ruminal protozoa count, when compared to a control
sample (no additive). Compared to the control diet, the addition of 30 µl eugenol reduced
total in vitro GP by 28%, CH₄ production by 38%, OM degredation by 18%, and
protozoal count (10⁵/ml) by 30.3%. In terms of total GP, as well as CH₄ production and
OM degradation, eugenol preformed very similarly to the positive control, monensin, with no significant difference being detected between the two.

**Advantage of Active Ingredient Encapsulation During Rumination (2.6)**

The main use of encapsulation in the food and pharmaceutical industries is the protection of sensitive active ingredients from an environment with the potential to physically, oxidatively, enzymatically, or microbially degrade the encapsulated bioactive (Emanuele, 2006). In many cases, the encapsulation technology serves as a system capable of controlling the time and location in which the active ingredient is released, allowing for specific, targeted ingredient delivery (Mamvura et al., 2014). The rumen, a large, anerobic, acidic chamber that is home to billions of ruminal bacteria, serves as a perfect example of an environment in which encapsulation technology could be employed to allow for the survival and targeted enteric delivery of an active ingredient.

Over the last decade, much interest has been turned towards the use of encapsulation in animal feed and animal nutrition industries for the purpose of improving cow performance (eg. average daily gain, reproduction, milk production) and altering rumen fermentation parameters (eg. volatile fatty acid [VFA] production, CH₄ emissions) (Emanuele, 2006; Mamvura, et al., 2014). Past studies have focused on the encapsulation of a number of different active ingredients. Additives such as nitrate and anti-microbial organic acids (eg. fumeric acid) have been encapsulated with the purpose of altering the VFA profile and reducing ruminal methane emissions (Wood et al., 2009; Mamvura et al., 2014). Vitamins (eg. ascorbic acid, choline) that are highly rumen degradable and sensitive to heat, moisture and oxidation have been encapsulated to alleviate degradation
during feed processing and rumination while ultimately improving liver function, milk yield and calf growth (Garrett et al., 2005; Cooke et al., 2007). Polyunsaturated fatty acids (e.g. conjugated linoleic acid) have also been encapsulated with the purpose of protecting the fatty acids from ruminal hydrogenation and improving the postruminal bioavailability (Perfield et al., 2004).

**In Vitro Rumination Methods (2.7)**

In an effort to estimate relative forage digestibility in a controlled, laboratory setting, *in vitro* techniques were established as far back as the 1950’s that utilize rumen fluid and artificial rumen fermentation vessels to predict *in vivo* digestibility parameters (Marten & Barnes, 1980). There are, for the most part, five diagnostic methods used to predict the energy digestibility of ruminant feeds: 1) the Tilley and Terry (1963) two-stage rumen fluid and acid-pepsin *in vitro* digestion (or modifications thereof), 2) *in situ* nylon-bag incubations within a cannulated rumen, 3) *in vitro* nylon-bag incubations in rumen-fluid filled fermentation jars, 4) purified cellulose fungal enzymatic *in vitro* fermentation system, and 5) *in vitro* gas-style incubations (Marten & Barnes, 1980; Makkar, 2004).

There are a number of factors that could potentially represent sources of variation when estimating digestibility with *in vitro* methods of ruminal fermentation. The rumen inoculum signifies the most significant potential discrepancy with *in vitro* systems, where source, preparation, sampling time and necessary anaerobic environment of the rumen fluid all represent sources of possible variation. As well, characteristics of the buffer solution, such as pH and ratio of medium to inoculum, could alter the microbial activity
during an *in vitro* incubation. Lastly, the environmental conditions, such as incubation temperature and oxygen exposure, could potentially influence the incubation and bias the output data (Marten & Barnes, 1980; Makkar, 2004).

**Cabinet-Style Nylon-Bag Incubation Method (2.7.1)**

While the *in situ* nylon-bag method of evaluating digestibility of feed samples has been widely used and trusted as an applicable and accurate estimation of *in vivo* rumination, it is a highly labour intensive and time-consuming procedure that, for validity, requires at least 3 ruminally cannulated animals (Makkar 2004; Trujillo *et al.*, 2010). However, *in vitro* nylon-bag incubations done in cabinet-style fermentation vessels (eg. ANKOM’s Daisy™ Incubator; ANKOM Tech., Macedon, NY, USA) are capable of providing reliably accurate estimates of *in vitro* dry matter disappearance (IVDMD) and neutral detergent fibre (NDF) disappearance of feed substrates, all while saving on time, labour, feed, and surgically-modified animals (Trujillo *et al.*, 2010).

The procedure typically involves weighing empty 25 µm porosity filter bags, filling the bags with a small amount of sample (0.25-0.5 g), heat-sealing the bags and placing them inside incubation jars (~25 bags per jar). A 39 °C buffer medium with a pH of 6.8 is then added to the digestion jars. Rumen inoculum is then collected, pooled from the donor cows, blended, purged with CO₂, filtered through four layers of cheesecloth and added to the digestion jar (1:4 ratio of inoculum to medium). The jars are purged with CO₂, sealed tightly and incubated for 48 hr at 39°C. Following the incubation, the jars are drained of fluid and the bags are washed with tap water then dried and re-weighed to determine DM disappearance. When performing *in vitro* true digestibility
(IVTD) measurements, the microbial debris is removed prior to re-weighing by washing bags with a neutral detergent solution via an NDF procedure (ANKOM Tech., 2005).

While research has shown that the in vitro nylon-bag incubations underestimate the in situ method for both dry matter (DM) and NDF, the advantages of the in vitro procedure (eg. repeatability, simplicity, practicality, etc.) make it a suitable and useful tool when comparing degradation and disappearance of feed substrates (Trujillo et al., 2010).

**Gas-Style Incubation Method (2.7.2)**

A considerable amount of gas (CO₂ and CH₄) is produced during fermentation and rumination, with significant contributions coming from the synthesis and usage of VFAs. These gases are disposed of via respiration, as well as a process called eructation where esophageal contractions hold food particles away from the esophagus to allow the built-up fermentative gases to be expelled (van Houtert, 1993; Russell & Rychlik, 2001). Direct gas production (GP) occurs only during the fermentation of a substrate to acetate and butyrate, as well as from methanogenic process that follows the production of these VFAs. Gas produced from substrate fermentation to propionate will be minimal and will be produced indirectly, via bicarbonate buffering (Makkar, 2004). Figure 2-4 illustrates the stoichiometry of VFA production from the fermentation of glucose while outlining the direct GP pathways via acetate and butyrate formation as well as methanogenesis.

| A) 1 mol Glucose + 2H₂O → 2 Acetate + 2CO₂ + 4H₂ |
| B) 1 mol Glucose + 2H₂ → 2 Propionate + 2H₂O |
| C) 1 mol Glucose → 1 Butyrate + 2CO₂ + 2H₂ |
| D) CO₂ + 4H₂ → CH₄ + 2H₂O |

*Figure 2-4. Stoichiometry of VFA production from glucose fermentation (A/B/C) and CH₄ generation via methanogenesis (D) (Stoichiometry from Wolin, 1960).*
Proportional shifts in VFA production will be create evident shifts in GP. Intake of a feed source that ferments more readily to acetate (eg. slowly digestible carbohydrate) will result in an increase in GP and vice versa for a feed source that more readily ferments to propionate (eg. rapidly digestible carbohydrate) (Makkar, 2004). Automated gas-fermentation systems monitor the extent and rate of organic matter degradation by tracking both direct and indirect gas production to give an accurate measurement of all the products of fermentation (eg. VFAs, microbial biomass, CO₂, and CH₄) (Makkar, 2004). By combining the *in vitro* GP system with mathematical models that calculate the kinetics of the microbial fermentation, researchers are able to build degradation profiles that reduce total GP down to two pools: 1) the fast-pool of rapidly fermenting, soluble carbohydrates (eg. sugar, starch, soluble fiber [pectin]), and 2) the slow-pool of gradually fermenting, insoluble carbohydrates (eg. cellulose and hemicellulose) (Johnston & Tricarico, 2007; Plaizier & Li, 2013). The differentiation between pools can help characterize feed sources in terms of nutritive value (eg. VFA profile and estimated microbial biomass yield) (Johnston & Tricarico, 2007).

**Research Rationale and Objectives (2.8)**

While a body of literature exists investigating the use of gel encapsulation technology as a delivery mechanism for bioactive ingredients, very little research has been directed towards exploring the use of these encapsulation techniques to safeguard an AI from ruminal degradation in order to deliver the bioactive to the blood stream of a dairy cow for utilization by the mammary glands. This study was conducted for the purpose of laying the groundwork for the natural fortification of milk products by feeding medicinal and therapeutic AIs to dairy cows while using natural, economical, versatile
gels as a target-specific delivery system for mammary gland nutrient-deposition. As well, the design and formulation of the gel complex used for this study was based around the potential delivery of both hydrophobic and hydrophilic AIs.

The objectives herein were to select an ideal organogelator and oil combination for rumen-protection, to define an optimized method of creating an emulsified complex comprised of the organogel and NaAlg, and ultimately, to examine – via 48 hr *in vitro* ruminal nylon-bag and gas fermentation incubations – the use of this unique gel complex to protect dried and ground HB from ruminal degradation. It was hypothesized that by encapsulating an AI within a protective gel complex, microbial fermentation and ruminal degradation of that encapsulated AI can be significantly reduced.
References (2.9)


Chapter 3.

In vitro ruminal degradation of holy basil (Ocimum sanctum) within an alginate gel and dispersed wax organogel droplets

Under review by, and formatted to suit, the Journal of the Science of Food and Agriculture

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ABSTRACT (3.1)

BACKGROUND: The study’s aim was to develop an encapsulation technology capable of loading either polar or apolar active ingredients, in this case ground holy basil leaves, that resists degradation throughout a 48 hr in vitro ruminal incubation. A rice-bran wax and canola oil organogel was homogenized with a sodium alginate solution to create a low viscosity oil-in-water emulsion, followed by a secondary gelation process initiated by calcium salts.

RESULTS: The organogel was utilized for the prospective delivery of hydrophobic active ingredients, and for its stability (dry matter disappearance of <7 %) during the primary 48 hr digestibility trial. The final gel complexes underwent a secondary in vitro incubation to determine their degradation rates and ability to retain the encapsulated active ingredient over a 48 hr fermentation period. Active ingredient retention rates for the 2 and 4% alginate gels were 72.5 and 90 %, respectively.

CONCLUSION: Following a 48 hr period of ruminal fermentation, dry matter disappearance of the encapsulated holy basil was significantly decreased, compared to unprotected holy basil. This study lays the groundwork for the natural fortification of milk products by feeding medicinal active ingredients to dairy cows using natural, versatile gels as a target-specific delivery system for mammary gland nutrient-deposition.
**Keywords:** holy basil (*Ocimum sanctum*); rumination; sodium alginate; encapsulation

**INTRODUCTION (3.2)**

Pregastric fermentation in ruminants stands as a roadblock for the delivery of ingested compounds into the blood. When there is a desire to fortify milk with functional ingredients to increase its value, encapsulation techniques to protect active ingredients (AIs) against rumen degradation constitute one approach to improve efficiency of transfer of the AI into milk.

As a gelation agent, sodium alginate (NaAlg) is versatile and innocuous, and the stable polymeric gel matrix it forms can safeguard encapsulated AIs from external stressors.\(^1\)\(^2\) Initiation of alginate gelation by Ca\(^{2+}\) can occur in one of two ways: externally, at a neutral pH, or internally, via acidification of the medium.\(^1\)\(^2\) External gelation involves administration of a water-soluble Ca\(^{2+}\) source (eg. CaCl\(_2\)•2H\(_2\)O) that immediately activates gelation via cationic interaction when added to a NaAlg solution. Alternatively, internal gelation occurs when a drop in pH causes the release of Ca\(^{2+}\) from an insoluble salt (eg. CaCO\(_3\)) already present in the NaAlg medium. Once liberated from its inactive form, the Ca\(^{2+}\) is free to interact with the NaAlg solution.\(^1\)\(^2\) This study utilized two stages of calcium-activated gelation: rapid and immediate (external) gelation via CaCl\(_2\)•2H\(_2\)O, as well as a continuous (internal) gelation via gradual liberation of Ca\(^{2+}\) from insoluble CaCO\(_3\) once the gel comes in contact with rumen fluid, causing a drop in pH, during *in vitro* incubation.

By incorporating a wax-in-oil (organogel) complex into the gel product, the potential exists to encapsulate hydrophobic, lipid-soluble AIs within the gel-matrix, in addition to hydrophilic, water-soluble AIs. Organogels are formed via low molecular-mass organogelators (LMOGs) that are dissolved in an organic liquid. The liquid is
heated to a temperature above the melting point of the organogelator to create a solution. Once this solution has been cooled to a temperature suitable for a solution-to-gel transition to occur, the organogelator molecules form a self-assembled fibrillar network (SAFiN) that is stabilized through non-covalent interactions and traps the organic solvent inside. Organogels can form in a highly effectively manner with a very low LMOG concentration, in many cases less than 2 wt%. 

Holy basil (HB; *Ocimum sanctum*), also known as Tulsi, is an aromatic plant cultivated across South Asia. The essential oils and abundance of secondary metabolites in HB endow it with a number of medicinal, health-promoting properties, including anti-stress, anti-oxidative, anti-ulcerogenic, and anti-microbial/bacterial actions. Along with its effectiveness in treating various ailments and preventing potential deficiencies, HB is also regarded as being economical and widely available. 

The objectives herein were to select an ideal organogelator and oil combination for rumen-protection, to define an optimized method of creating an emulsified complex comprised of the organogel and NaAlg, and ultimately, to examine – via 48 hr *in vitro* rumen fluid incubations – the use of this unique complex to protect dried and ground HB from ruminal degradation.

**MATERIALS AND METHODS (3.3)**

**Wax-in-oil sample preparation (3.3.1)**

Three organogelators were considered for the dispersed phase of a final emulsion: 12-hydroxystearic acid (12-HSA), candelilla wax (CW), and rice-bran wax (RBW). They were supplied by Koster Keunen, (Watertown, CT, USA) and were chosen based on the criteria that they are non-toxic, economical, and gel at relatively low concentration. For each organogel, the respective organogelator (12-HSA, CW, RBW) was added to either
canola oil or soybean oil at 2 wt% and then heated to 85 °C until complete dissolution occurred. Once the organogelator was dissolved, the solution was cooled to room temperature (20 °C) for 24 hrs. The primary in vitro anaerobic-incubation analyzed all six organogels, in duplicate, to determine their respective 24 and 48 hr dry matter (DM) disappearance rates.

**Emulsion preparation (3.3.2)**

Sodium alginate (*Macrocystis pyrifera*), emulsifiers (Span80/Tween80), and Ca$^{2+}$ salts (CaCO$_3$/ CaCl$_2$•2H$_2$O) were obtained from Sigma Aldrich (Oakville, ON, Canada). 2 and 4 % (w/v) NaAlg aqueous solutions were prepared by mixing NaAlg powder with 50 °C de-ionized water in a blender for 5 min. 10.8 and 5.4 g of NaAlg powder were added to 270 ml water for the 4 and 2 % solutions, respectively. The solutions were stored at room temperature (20 °C) for 24 hr to ensure for complete deaeration. The NaAlg solutions were heated in a water bath for 10 min at 85 °C before being added into 18 ml volumes containing 50 % Span 80 and 50 % Tween 80. 2 wt% RBW was dispersed in canola oil and heated at 85 °C in a water bath until complete dissolution (approximately 20 min). 90 ml of the RBW solution was added to the NaAlg/emulsifier solution to produce a 3:1 ratio of NaAlg:RBW and the mixture was homogenized (Ultra-Turrax T25 homogenizer, IKA Works Inc., Wilmington, NC, USA) for 5 min. The homogenized mixtures were then emulsified using the M-110EH-30 Pilot Production Microfluidizer (Microfluidics, Westwood, MA, USA) for 4 cycles at 10,000 PSI. The emulsion was collected after 4th cycle and cooled at 20 °C for 2 hrs.

Four separate batches of Ca$^{2+}$ slurry were prepared by adding 0.8 g CaCO$_3$ and 0.08 g CaCl$_2$•2H$_2$O to 100 ml volumes of 80 °C, deionized water. 9.75 g of ground HB
powder (*Ocimum sanctum* leaves, Davidson’s Organic Teas, Herbs, Cocoa, and Spices, Sparks, NV, USA) was added to two batches of the calcium slurry. All four batches of Ca$^{2+}$ slurry were then added at a 1:1 (v:v) ratio into separate allotments of the NaAlg/RBW emulsion to form the four gel complexes described in Table 3-1.

**Table 3-1.** Descriptions of the five samples tested in the secondary 48 hr in vitro rumination incubation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>100ml 4 % NaAlg/RBW emulsification + 100 ml Ca$^{2+}$slurry + 5% HB</td>
<td>4-EHB</td>
</tr>
<tr>
<td>100ml 2 % NaAlg/RBW emulsification + 100 ml Ca$^{2+}$slurry + 5% HB</td>
<td>2-EHB</td>
</tr>
<tr>
<td>100ml 4 % NaAlg/RBW emulsification + 100 ml Ca$^{2+}$slurry</td>
<td>4-NHB</td>
</tr>
<tr>
<td>100ml 2 % NaAlg/RBW emulsification + 100 ml Ca$^{2+}$slurry</td>
<td>2-NHB</td>
</tr>
<tr>
<td>Only dried and ground HB</td>
<td>OHB</td>
</tr>
</tbody>
</table>

*In vitro* rumen degradation (3.3.3)

*In vitro* testing was completed under CO$_2$ in an ANKOM DAISY$^\text{II}$ incubator cabinet (ANKOM Tech., Macedon, NY, USA). Two separate trials were run, a primary incubation with the six organogel complexes (24 and 48 hr), and a secondary incubation with the five samples outlined in Table 3-1 (48 hr). 0.4 g of each sample was added to F57 nylon filter bags (ANKOM Tech., Macedon, NY, USA) and bags were heat-sealed. One sealed, empty filter bag was included as a blank. Rumen fluid was collected from lactating, adult, rumen-fistulated cows fed a 60 %–forage total mixed ration (TMR). The inoculum was blended and strained through four layers of cheesecloth into a flask pre-heated to 39 °C. A phosphate buffer (pH 6.8) was prepared according to Marten and Barnes. $^{10}$ 400 ml of inoculum (pH 5.29) was added to each digestion jar and diluted 1:4 with the buffer. The nylon-bags were put into the digestion jars; the jars were purged with CO$_2$, sealed, and incubated at 39 °C for 48 hr. For the primary incubation, the organogel sample bags were removed at 24 and 48 hrs. Each sample and time-span for both incubations was run in duplicate. After incubation, the bags were washed with tap water and dried at 20 °C to determine *in vitro* DM disappearance. $^{11}$
Statistical Analysis (3.3.4)

Effects of HB form (absent, non-encapsulated and encapsulated), NaAlg level (0, 2 and 4 %), and the form × level interaction, were analyzed with Proc GLM of SAS (Version 9.2 SAS Institute Inc., Cary, NC, USA). Treatment means were separated by Tukey adjusted multiple comparisons with significance declared at $P \leq 0.05$.

RESULTS AND DISCUSSION (3.4)

Of the six organogels studied, the RBW/canola oil combination degraded, on average, 10.5 % less than the other formulations after 24 hr, and 22.5 % less after 48 hr (Figure 3-1). While the rumen-stability of the RBW was desirable, another advantage of incorporating an organogel into the final gel was the potential to encapsulate and deliver lipid-soluble AIs. The capability of encapsulating hydrophobic and/or hydrophilic AIs within the final complex allows delivery of a much wider variety of AIs.

![Figure 3-1](chart.png)

**Figure 3-1.** Duplicate-avg. DM disappearance (%) for each of the six organogels after 24 and 48 hr of the primary in vitro rumination incubation. *The organogelator and oil chosen for the final gel emulsion.

The secondary in vitro, ANKOM digestion trial yielded 48 hr extents of ruminal DM degradation of the 2 and 4 % gel complexes, both with (EHB) and without (NHB)
encapsulated HB, as well as for the sample of only dried and ground HB (OHB). The difference between the DM disappearance of the EHB and NHB samples represented the loss of the encapsulated AI. The OHB sample provided a baseline value for the ruminal DM degradation of un-encapsulated HB. Comparing the DM disappearance (Figure 3-2) of EHB and NHB gels revealed that, while a significant portion of all four gel complexes was lost during the incubation (avg. loss of 85.3 %), the loss of the encapsulated HB itself (AI) was far less (avg. loss of 18.8 %).

The 2- and 4-EHB gels began the incubation with 20 mg of encapsulated AI per 400 mg sample (5 % HB w/w). The differences between the DM disappearance of the EHB and NHB samples were 14.5 and 18 mg, for the 2 and 4 % gels respectively. These amounts represent the HB remaining in the DM residue of each respective gel. When compared to the 20 mg that each of the EHB samples began the incubation with, AI-retention of the 2 and 4 % gels was 72.5 and 90 %, respectively, after 48 hr. The modest

<table>
<thead>
<tr>
<th>Treatment</th>
<th>48 hr % DM disappearance</th>
<th>% AI-retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>OHB</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>2-NHB</td>
<td>85</td>
<td>90</td>
</tr>
<tr>
<td>4-NHB</td>
<td>87</td>
<td>95</td>
</tr>
<tr>
<td>2-EHB</td>
<td>88</td>
<td>97</td>
</tr>
<tr>
<td>4-EHB</td>
<td>89</td>
<td>100</td>
</tr>
</tbody>
</table>

**Figure 3-2.** Duplicate-avg. DM disappearance (%) and AI (HB) retention (%), for the five samples (Table 3-1) tested in the secondary 48 hr *in vitro* rumination incubation procedure.
loss of the encapsulated AI (27.5 and 10% for the respective 2 and 4% gels), compared to the degradation of the gel complexes themselves, indicates that the encapsulation process was effective in protecting the AI from substantial ruminal degradation. Our finding is that, on average, these gels can protect approximately 81% of an encapsulated AI from microbial breakdown within the rumen.

Table 3-2 shows a significant difference in AI retention between the dried and ground HB that was incubated “as-is” (with no protective encapsulation) and that same HB enveloped within the NaAlg/RBW gel. This indicates that the form of HB significantly affected its degradation rate. After 48 hr, approximately 45% (178 of 400 mg) of the OHB sample had been degraded, translating to an AI-retention of 55.4%, compared to 72.5 and 90% AI retention of the 2- and 4-EHB samples, respectively. Thus, the gel-encapsulation increased AI-retention by an average of 25.9%. Table 3-2 also indicates that the 4% alginate gel was significantly more effective in protecting the AI against ruminal degradation than the 2% gel, with the AI-retention of the 4-EHB sample being 17.5% higher than the 2-EHB sample. These results suggest that, for the purpose of optimizing AI-retention throughout a 48 hr ruminal fermentation period, HB should be encapsulated within a 4% NaAlg gel.

Table 3-2. Tukey letters and respective means for each sample (A-E; n=2) from Table 3-1 as well as SEM and P-values for sample Form, Level and Form x Level interaction.

<table>
<thead>
<tr>
<th></th>
<th>4-EHB</th>
<th>2-EHB</th>
<th>4-NHB</th>
<th>2-NHB</th>
<th>OHB</th>
<th>SEM</th>
<th>F-value</th>
<th>L-P-value</th>
<th>Form x Level-P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI Retention</td>
<td>$90^a$</td>
<td>$72.5^b$</td>
<td>$0^d$</td>
<td>$0^d$</td>
<td>55.375$^c$</td>
<td>2.761</td>
<td>$&lt;0.001$</td>
<td>0.0249</td>
<td>0.0249</td>
</tr>
</tbody>
</table>

*Means with difference superscripts differ (P <0.05).

A number of studies have demonstrated the inhibitory actions of HB against growth and activity of both Gram-positive and Gram-negative bacteria.\(^8,9\) Oil extracted from the HB leaves (\textit{Ocimum sanctum}) is comprised of approximately 71% eugenol and
20 % methyl-eugenol, which are believed to be largely responsible for its anti-microbial
effect.\textsuperscript{8,9,12,13} This anti-microbial action may explain how the DM disappearance of the
dried and ground HB, with no protective encapsulation, was only 45.2 % after a 48 hr
ruminal fermentation period. In comparison, dried and ground barley, wheat bran, pea
grain, and alfalfa hay typically exhibit 48 hr ruminal DM disappearance rates of 82.8,
77.2, 88.6, and 75.4 %, respectively.\textsuperscript{14,15} The low degradability of HB suggests that HB
and its secondary metabolites inhibit the microbial breakdown of its organic structure.

**CONCLUSIONS (3.5)**

An organogel complex comprised of RBW and canola oil is ideal when
considering its rumen stability and the prospective delivery of hydrophobic AIs, and that
encapsulation technology comprised of an organogel and NaAlg emulsion significantly
decreased the DM disappearance of the encapsulated HB following a 48 hr period of
ruminal fermentation, when compared to unprotected HB. Finally, as the 4-EHB sample
retained 17.5 and 35.2 % more HB then the 2-EHB and OHB samples, respectively, the
4 % NaAlg gel is the most suitable option for supplementary studies.

**CONFLICT OF INTEREST (3.6)**

None declared.

**ACKNOWLEDGEMENTS (3.7)**

The authors are grateful for the financial support received from of the Dairy
Farmers of Ontario (Mississauga, ON, Can) and the Ontario Centres Of Excellence
(Toronto, ON, Can).
REFERENCES (3.8)


In vitro ruminal gas production of alginate gel encapsulated holy basil (Ocimum sanctum L.) and the potential antimicrobial effects of holy basil’s active secondary metabolites.

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ABSTRACT (4.1)

This study was conducted to investigate the ruminal degradation resistance and gas production throughout a 48 hr in vitro gas-fermentation incubation of an encapsulation technology capable of loading either polar or apolar active ingredients, in this case dried, finely ground holy basil leaves. Gel formulation was preformed by homogenizing a rice-bran wax and canola oil organogel with a sodium alginate solution to create a low viscosity oil-in-water emulsion, followed by a secondary gelation process initiated by calcium salts. The organogel was utilized for the prospective delivery of hydrophobic active ingredients. The ruminal stability of the final gel complex, as well as the discrete stabilities of the individual gel constituents, was tested using an in vitro gas-fermentation system to determine the products’ fermentative gas production (CO₂ and CH₄) over a 48 hr incubation period.

Interestingly, the gel samples with 5% encapsulated holy basil produced significantly more fermentative gases compared the samples of the same gel complex that had the 5% holy basil added on top of the gel immediately before the incubation. The significant difference was seen both in observed gas production rates (62.75 % avg. difference) as well as the predicted gas production curves (95 % avg. difference) that were modeled with an adapted Michaelis-Menten equation. The anti-microbial actions of
holy basil and its secondary metabolites are speculated to be potentially responsible for the variation in gas production between the two sample treatments.

The combined gas chromatography-mass spectrometry analysis identified 36 % concentration of eugenol using a single ion monitoring scan and GC-MS grade eugenol standard. A GC full scan tentatively identified appreciable amounts of methyl eugenol (25 %), caryophyllene (18 %), camphor (9 %), and naphthalene (4 %). Eugenol and methyl-eugenol have been recognized as the most influential active compounds found in holy basil in terms of its anti-microbial actions. The gas production and GC-MS results support previous research attributing anti-microbial and rumen-modifiable properties to holy basil and its secondary metabolites. This study lays further groundwork for the natural fortification of milk products by feeding medicinal and therapeutic active ingredients to dairy cows while using natural, economical, versatile gels as a target-specific delivery system for mammary gland nutrient-deposition.

**Keywords:** holy basil (*Ocimum sanctum*); anti-microbial; encapsulation; gas production

**Introduction (4.2)**

Pregastric fermentation in ruminants stands as a significant roadblock for the delivery of ingested compounds into the blood. When there is a desire to fortify milk with functional ingredients to increase its value, encapsulation techniques to protect active ingredients (AIs) against rumen degradation constitute one potential approach to improve efficiency of transfer of the AI into milk. An emulsified gel complex, consisting of both water- (sodium alginate) and oil-based (rice-bran wax) gel phases, was previously formulated by Templeman et al. (*Chapter 3*) for the purpose of safeguarding AIs from ruminal fermentation while maintaining the potential to encapsulate and deliver polar or
apolar AIs. This ruminal stability of this formulation was previously analyzed via *in vitro* nylon-bag style incubations and, in terms of dry matter (DM) disappearance, demonstrated AI retention rates of 73 and 90 % for the 2 and 4 % NaAlg-based gels, respectively, following a 48 hr incubation period (Templeman *et al*. *Chapter 3*).

As a gelation agent, sodium alginate (NaAlg) is versatile and innocuous, and the stable gel matrix it forms can capably encapsulated and safeguard AIs from external stressors (Funami *et al*., 2009; Lupo *et al*., 2014). Extracted from the cell walls of brown seaweed, NaAlg is considered an ideal candidate for the encapsulation and delivery of hydrophillic active ingredients or volatile bioactives such as plant polyphenols (Florián-Algarín & Acevedo, 2010; Lupo *et al*., 2014). Alginate is a linear, unbranched copolymer of alternating blocks of (1-4)-linked β-D-mannuronic acid and α-L-guluronic acid residues. When exposed to divalent cations, such as Ca$^{2+}$, the ratio and distribution of these uronic residues influence strength and elasticity of the alginate gel formed (Funami *et al*., 2009; Florián-Algarín & Acevedo, 2010; Khoury *et al*., 2014).

Initiation of alginate gelation by Ca$^{2+}$ can occur in one of two ways: externally, at a neutral pH, or internally, via acidification of the medium (Poncelet *et al*., 1995; Funami *et al*., 2009; Lupo *et al*., 2014). External gelation involves administration of a water-soluble Ca$^{2+}$ source (eg. CaCl$_2$•2H$_2$O) that immediately activates gelation via cationic interaction when added to a NaAlg solution. Alternatively, internal gelation occurs when a drop in pH causes the release of Ca$^{2+}$ from an insoluble Ca$^{2+}$ salt (eg. CaCO$_3$) already present in the NaAlg solution. Once liberated from its inactive form, the Ca$^{2+}$ is free to interact with the NaAlg itself (Funami *et al*., 2009; Lupo *et al*., 2014). This study utilized two stages of gelation: rapid and immediate (external) gelation via CaCl$_2$•2H$_2$O, as well
as a continuous (internal) gelation via gradual liberation of Ca$^{2+}$ from insoluble CaCO$_3$ once the gel comes in contact with rumen fluid, causing a drop in pH, during *in vitro* incubation.

By incorporating a wax-in-oil (organogel) complex into the gel product, the potential exists to encapsulate hydrophobic, lipid-soluble AIs within the gel-matrix, in addition to hydrophilic, water-soluble AIs. Organogels are formed via low molecular-mass organogelators (LMOGs) that are dissolved in an organic liquid. Once the liquid is heated above the melting point of the organogelator, a solution is created that, upon cooling, undergoes a solution-to-gel transformation (Abdallah & Weiss, 2000). During this transition, the organogelator molecules form a self-assembled fibrillar network (SAFiN) that is stabilized through non-covalent interactions to ultimately trap the lipophilic AI and the organic solvent inside. Organogels can form in a highly effective manner with a relatively low LMOG concentration, in many cases less than 2 wt% (Abdallah & Weiss, 2000). Rice-bran wax (RBW) is a rumen-stable, non-toxic, economical, and readily available organogelator that displays high crystallinity, allowing for it to gel an organic liquid at a relatively low concentration (Dassanayake *et al*., 2009). Rice-bran wax and canola oil was determined to be an ideal organogelator and organic liquid combination for the oil-phase portion of the final gel complex, as it has been shown to degrade, on average, 23 % less than other comparable organogelators after a 48 hr nylon-bag rumen incubation (Templeman *et al*., Chapter 3).

Holy basil (HB; *Ocimum sanctum*), also known as Tulsi, is an aromatic plant widely cultivated across South Asia. While it is believed to be religiously sacred (Dharmani *et al*., 2004), the secondary metabolites in HB endow it with a number of
medicinal, health-promoting properties, including anti-stress (Gupta et al., 2007; Singh et al., 2012), anti-oxidative (Vrinda & Devi, 2001), anti-ulcerogenic (Dharmani et al., 2004), and anti-microbial/bacterial actions (Gupta et al., 2002; Viyoch et al., 2006). Along with its effectiveness in treating various ailments and preventing potential deficiencies, HB is also regarded as being economical and widely available (Singh et al., 2012).

A dairy cow’s rumen is a large anaerobic chamber that works to break down ingested plant material via a microbically active fermentation process (Russell & Rychlik, 2001). The rumen houses billions of bacterial, protozoal, and fungal microbes (>10^{10} cells/g rumen content) that work symbiotically with the cow to digest the soluble (eg. β-glucans and pectins) and insoluble (eg. cellulose and hemicellulose) fibrous components of dietary forages (Huhtanen et al., 2006). The ruminal microbes ferment carbohydrate substrates to form short-chain, volatile fatty acids (VFAs). These VFAs primarily consist of acetic, propionic, and butyric acids.

Direct gas production (GP) occurs only during the fermentation of a substrate to acetate and butyrate, as well as from methanogenesis that follows the production of these VFAs. The synthesis and usage of these VFAs by the cow contribute a considerable amount of the gas produced during fermentation that is disposed of via either expiration or eructation (Russell & Rychlik, 2001; Ungerfeld & Kohn, 2006). Gas produced from substrate fermentation to propionate will be minimal and will be produced indirectly, via bicarbonate buffering (Makkar, 2004). Proportional shifts in VFA production will create evident shifts in GP. Intake of a feed source that ferments more readily to acetate (eg. neutral detergent fiber; NDF) will result in an increase in GP and vice versa for a feed source that more readily ferments to propionate (eg. starch) (Makkar, 2004).
The objective herein was to investigate – via 48 hr *in vitro* gas-fermentation analysis – the use of this unique organogel and NaAlg emulsified complex to protect dried and ground HB from ruminal degradation. It is hypothesized that by encapsulating an AI within a versatile, protective gel complex, microbial fermentation and ruminal degradation of that AI can be significantly reduced.

**Materials and Methods (4.3)**

**Emulsification Formulation (4.3.1)**

The sodium alginate (*Macrocystis pyrifera*; 87.9 % purity), emulsifiers (Span80/Tween80), and Ca$^{2+}$ salts (CaCO$_3$/ CaCl$_2$•2H$_2$O) were obtained from Sigma Aldrich (Oakville, ON, Canada). Rice-bran wax, supplied by Koster Keunen Inc., (Watertown, CT, USA). 2 wt% of RBW was used as the organogelator, and canola oil as the organic liquid, for the dispersed phase of the final emulsification.

2 and 4 % (w/v) NaAlg aqueous solutions were prepared by mixing NaAlg powder with 50 °C de-ionized water in a blender for 5 min. 10.8 and 5.4 g of NaAlg powder were added to 270 ml water for the 4 and 2 % solutions, respectively. The solutions were stored at room temperature (20 °C) for 24 hr to ensure complete deaeration. The NaAlg solutions were heated in a water bath for 10 min at 85 °C before being added into 18 ml volumes containing 50 % Span 80 and 50 % Tween 80. 2 wt% RBW was dispersed in canola oil and heated at 85 °C in a water bath until complete dissolution (~20 min). 90 ml of the RBW solution was added to both 2 and 4% NaAlg/emulsifier solutions to produce 3:1 ratios of NaAlg:RBW and the mixtures were each homogenized (Ultra-Turrax T25 homogenizer, IKA Works Inc., Wilmington, NC, USA) for 5 min. The
homogenized mixtures were then emulsified using the M-110EH-30 Pilot Production Microfluidizer (Microfluidics, Westwood, MA, USA) for 4 cycles at 10,000 PSI. The emulsions were collected after 4th cycle and cooled at 20 °C for 2 hr. The 2 and 4% NaAlg emulsions were both allocated into 3 subsamples in preparation for the calcium salt addition.

**Calcium Salt Gelation Activation (4.3.2)**

Six separate batches of \( \text{Ca}^{2+} \) slurry were prepared by adding 0.8 g CaCO\(_3\) and 0.08 g CaCl\(_2\)•2H\(_2\)O to 100 ml volumes of 80 °C, deionized water. 9.75 g of ground HB powder (\textit{Ocimum sanctum} leaves, Davidson’s Organic Teas, Herbs, Cocoa, and Spices, Sparks, NV, USA) was added to two batches of the calcium slurry. All six batches of \( \text{Ca}^{2+} \) slurry were then added at a 1:1 (v:v) ratio into separate allotments of the NaAlg/RBW emulsion to form the six gel complexes described in Table 4-1.

**Table 4-1.** Treatment descriptions, abbreviations, and inclusion-weights of the AI (HB) for each sample analyzed via 48 hr gas-fermentation.

<table>
<thead>
<tr>
<th>#</th>
<th>n</th>
<th>Sample Weight (g)</th>
<th>Treatment</th>
<th>Abbreviation</th>
<th>HB Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>0.4</td>
<td>4% NaAlg/RBW emulsification + ( \text{Ca}^{2+} ) slurry + 5% Encapsulated HB</td>
<td>4-EHB</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>3*</td>
<td>0.4</td>
<td>2% NaAlg/RBW emulsification + ( \text{Ca}^{2+} ) slurry + 5% Encapsulated HB</td>
<td>2-EHB</td>
<td>0.02</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.4</td>
<td>4% NaAlg/RBW emulsification + ( \text{Ca}^{2+} ) slurry + No HB</td>
<td>4-NHB</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>0.4</td>
<td>2% NaAlg/RBW emulsification + ( \text{Ca}^{2+} ) slurry + No HB</td>
<td>2-NHB</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>0.4</td>
<td>4% NaAlg/RBW emulsification + ( \text{Ca}^{2+} ) slurry + 5% HB added On-Top immediately prior to incubation</td>
<td>4-HBOT</td>
<td>0.02</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>0.4</td>
<td>2% NaAlg/RBW emulsification + ( \text{Ca}^{2+} ) slurry + 5% HB added On-Top immediately prior to incubation</td>
<td>2-HBOT</td>
<td>0.02</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>0.4</td>
<td>Only dried and ground HB</td>
<td>OHB</td>
<td>0.4</td>
</tr>
</tbody>
</table>

NaAlg, sodium alginate; HB, holy basil; g, grams; n, number; AI, active ingredient
*One replicate from 2-EHB trt. was considered irregular following incubation and removed from further analysis.

**In Vitro Gas Production (4.3.3)**
Seven samples (Table 4-1) were analyzed in triplicate via a rumen-fluid, batch culture, gas-fermentation system (RFS Technologies, Ottawa, ON, Canada). Rumen fluid was collected from, and pooled between, adult, lactating, rumen-fistulated cows producing a minimum of 30 kg/day of milk while on a diet consisting of at least 60% forage and 20% starch. Following a 6:45 am feeding, rumen fluid was collected at 9:30 am and filtered through layered cheesecloth. The fermentation trial began within 30 minutes of inoculum collection (Fermentrics™, 2013).

All samples and blank controls were incubated in triplicate in 250ml gas tight culture jars. Prior to buffer or sample addition, the digestion jars were heated in a water bath to 39 °C. This 39 °C temperature was maintained throughout the entire sample preparation, as well as throughout the 48 hr incubation. A Kansas State phosphate buffer was prepared according to the procedure outlined by Marten and Barnes (1980). Two solutions were prepared (A: KH$_2$PO$_4$, MgSO$_4$$\cdot$7H$_2$O, NaCl, CaCl$_2$$\cdot$2H$_2$O; B: Na$_2$CO$_3$, Na$_2$S$\cdot$9H$_2$O), and were combined in a 5:1 ratio of solution A to solution B. Solution B was then added in a drop-wise manner to the 5:1 mixture to adjust pH to 6.8. Each replicate jar was flushed with CO$_2$ to allow for the anaerobic addition of 80 ml of buffering medium. The jars were then sealed and pre-warmed in the water bath to maintain the 39 °C temperature prior to the addition of the sample and rumen inoculum. 0.4 g of each sample was weighed out and placed into 3 replicate jars containing the buffer solution. For the 2- and 4-HBOT treatments, 380 mg of the 2 and 4% NaAlg emulsifications (with the Ca$^{2+}$ slurry addition) were added to the buffer in their respective triplicate samples jars. Then, just prior to the inoculum addition, HB powder was added at the same 5% inclusion level (20 mg) that would be encapsulated within the 2- and 4-
EHB samples. Finally, 20 ml of rumen fluid (pH of 5.36) was added to the all the jars containing the buffer and sample. The jars were purged again with CO₂ and re-sealed. Three negative control vials (blanks) that included only the rumen fluid and buffer were then prepared for each of the samples analyzed with the same CO₂ flush method. All the digestion jars were returned to the 39°C water bath, had their individual gas detection sensors affixed to the sealed lids, and the fermentation process began. Pressure was recorded every 30 seconds for 48 hr.

**Extraction Procedure (4.3.4)**

Gas chromatography-mass spectrometry (GC-MS) grade methanol (MeOH) and 0.22 µm nylon syringe filters were both purchased from Sigma Aldrich Canada (Oakville, ON, Canada). Finely ground, dried HB leaves were extracted using 80% MeOH at a 1:5 (w/v) sample DM to solvent ratio. The mixture was vortexed for 30 seconds using an Analog Vortex Mixer (Fisher Scientific, Ottawa, ON, Canada) and then sonicated with an Ultrasonic 3510 Sonified Water Bath (Branson Ultrasonics, Danbury, CT, USA) for 1 h at 25°C. The extract was centrifuged in an IEC HN-SII centrifuge (Magnolia, TX, USA) at 2000 rpm for 10 min. The supernatant was carefully collected and filtered through a 0.22 µm nylon syringe filter. To evaporate solvent, the supernatant was placed in a water bath heated to 40 °C until the final volume was one-fourth the original volume. The extract was stored at 4 °C in preparation for analysis by GC-MS.

**Gas Chromatography–Mass Spectrometry Procedure (4.3.5)**

A GC-MS grade analytical standard of eugenol was purchased from Sigma Aldrich Canada (Oakville, ON, Canada). Analyses were performed using an Agilent 7890A gas chromatograph coupled to an Agilent 5975 MSD single quadrupole mass
analyzer (Agilent Technologies, Santa Clara, CA, USA). The analytes were separated using BR-S wax capillary column (30 m x 0.25 mm i.d., 0.25 mm film thickness), operating at 7 psi of column head pressure, resulting in a flow of 1.0 ml/min at a starting temperature of 40 °C (Bruker Biospin Corp., Billerica, MA, USA). 1 µl of the HB extract sample was introduced into the injection port at 250 °C in splitless mode. The temperature program was isothermal for 2 min at 40 °C, raised to 100 °C at a rate of 20 °C per min, then raised to 220 °C at a rate of 10 °C per min, and finally raised to 250 °C at a rate of 40 °C per min and held for 4 min. The transfer line to the mass spectrometer was maintained at 260 °C. The ionisation source temperature was 230 °C and the ions were obtained by electron impact ionization in positive ion mode at 70 eV, collecting data at a rate of 1 scan per 200 ms.

Statistical Analysis (4.4)

Gas Production Models (4.4.1)

Two equations were fitted to cumulative GP (G) using Solver, in Microsoft Excel (Microsoft Corporation, Mississauga, ON, Canada), to minimized the residual sum of squares between predicted and observed GP. *Equation 1* was a Michaelis-Menten equation (Groot *et al.*, 1996; Ellis *et al.*, 2016) adapted to determine monophasic, GP with no lagtime:

\[ G = \frac{A}{1 + (C/t)^B} \]

*Equation 1*

where G (ml/400mg substrate) is the cumulative gas production (GP), A is the asymptotic GP (ml/400mg substrate), B is the inflection point, C is the time at which half the asymptotic GP has been reached (T_{1/2}, min), and t is the incubation time (min).
2 was an exponential Mitscherlich equation (France et al., 2000), in which B represents the fractional degradation rate:

\[
G = A(1-e^{-(B(t-C))})
\]

Equation 2

Residual sums of squares were lower with Equation 1 than Equation 2, so only data from the Groot equation was analyzed. Values for G at 24 hr (t24) were estimated from fitted equations.

Effects of HB form (absent, non-encapsulated, added prior to incubation, and encapsulated), NaAlg level (0, 2 and 4%), and the form \times level interaction, were analyzed using Proc GLM of SAS (Version 9.2 SAS Institute Inc., Cary, NC, USA). Differences among the parameters A, B, C, and t24 were determined using Tukey adjusted multiple comparison procedure within the MEANS statement. Significance was declared at \( P \leq 0.05 \). As per Ellis et al. (2016), no blank bottle correction was made to avoid the potential negative gas production that can occur, especially at the early time points during the incubation period. As the intent was to analyze multiple time points over a wide time range, no blank correction was used for total GP to avoid distorted values.

**GC-MS Analysis (4.4.2)**

Gas chromatography quantification of eugenol was performed using single ion monitoring (SIM) for the major ion of eugenol (molar mass of 164.2 g/mol), with a calibration curve created using a purchased eugenol standard for reference. A full scan was run as well, which monitored every visible peak within the \( m/z \) range of 50-550, allowing for tentative identification of all observed compounds by comparing their mass spectra with those contained in the NIST/EPA/NIH-14\textsuperscript{th} edition (V. 2.2f) and Wiley-11\textsuperscript{th} edition
edition MS libraries (Thermo Fisher Scientific, Austin, TX, USA). The relative areas (%) of the individual constituents were determined based on the GC peak area (response).

Results (4.5)

Gas Production (4.5.1)

As shown in Table 4-2, the encapsulated samples (2- and 4-EHB) significantly differed from all the rest of the treatments in asymptotic (A) and 24 h GP (t24).

Compared to 4-HBOT, the 4-EHB samples had significantly higher asymptotic GP and 24 h GP, as well as a significantly longer time taken for half the asymptotic GP to be reached (C). These three parameters were also significantly higher for the 2-EHB versus the 2-HBOT samples.

Table 4-2. Tukey letters and respective means for each parameter of each sample (#1-6, Table 4-1) modeled with the Groot equation (Equation 1) as well as SEM, R^2, and P-values for sample Form, Level and Form x Level interaction.

<table>
<thead>
<tr>
<th></th>
<th>4-EHB</th>
<th>2-EHB</th>
<th>4-NHB</th>
<th>2-NHB</th>
<th>4-HBOT</th>
<th>2-HBOT</th>
<th>OHB</th>
<th>SEM</th>
<th>R^2</th>
<th>F P-value</th>
<th>L P-value</th>
<th>FxL P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A ml/0.4g</td>
<td>1035.3^a</td>
<td>1368^a</td>
<td>102.6^b</td>
<td>86.7^b</td>
<td>47.2^b</td>
<td>72.4^b</td>
<td>22.1^b</td>
<td>111.18</td>
<td>0.907</td>
<td>&lt;0.001</td>
<td>0.249</td>
<td>0.316</td>
</tr>
<tr>
<td>B</td>
<td>0.478^b</td>
<td>0.51^b</td>
<td>0.891^b</td>
<td>0.901^b</td>
<td>0.936^b</td>
<td>1.087^b</td>
<td>8.188^b</td>
<td>1.399</td>
<td>0.646</td>
<td>0.937</td>
<td>0.958</td>
<td>0.999</td>
</tr>
<tr>
<td>C hr</td>
<td>13420.4^a</td>
<td>21109^a</td>
<td>136.92^b/c</td>
<td>118.47^b/c</td>
<td>84.12^c</td>
<td>105.10^b/c</td>
<td>43.47^c</td>
<td>2637.2</td>
<td>0.799</td>
<td>&lt;0.001</td>
<td>0.273</td>
<td>0.329</td>
</tr>
<tr>
<td>t24 hr</td>
<td>50.103^a</td>
<td>41.282^a</td>
<td>17.906^b/c</td>
<td>17.586^b/c</td>
<td>11.066^b/c</td>
<td>19.427^b/c</td>
<td>7.971^c</td>
<td>1.924</td>
<td>0.967</td>
<td>&lt;0.001</td>
<td>0.876</td>
<td>0.004</td>
</tr>
</tbody>
</table>

EHB; encapsulated holy basil; NHB, no holy basil; OHB, only holy basil; HBOT, holy basil on-top; HB, holy basil; SEM, standard error of the mean; F, Form; L, Level; FxL, Form x Level interaction; n, number of samples.

Means with difference superscripts differ (P <0.05).

The form of the HB significantly affected its fermentative GP rate for asymptotic GP (A), 24 h GP (t24), and the time at which half the asymptotic GP was reached (C).

The largest disparity occurred at the maximum (asymptotic) level, where the EHB samples had, on average, a higher GP rate than the HBOT, NHB and OHB treatments by 95, 92, and 99 %, respectively. Within that range of asymptotic GP, the NHB samples, on average, produced 36 % more fermentative gases than the HBOT samples, and 88 %
more than the OHB samples. At the 24 hr mark, these EHB samples averaged a 67, 61, and 91 % higher GP rate than the respective HBOT, NHB, and HBO samples. From these same values, the NHB treatments produced an average of 14 and 78 % more fermentative gases than the HBOT and HBO treatments, respectively. It should be noted that the modeled “A” values (asymptotic/maximal GP) in Table 4-2 are consistently higher than the observed values (Figure 4-1) due to the fact that at 48 hr of incubation, the samples had not reached a defined GP plateau, thus were still producing fermentative gases once the 48 hr incubation period was completed.

Figure 4-1. Observed maximum (asymptotic) and t24 (GP at 24 hr of incubation) gas production for each sample incubated (triplicate-avg.).

**GC-MS (4.5.2)**

Varying concentrations of the eugenol standard (0.5 mg/ml to 0.03125 mg/ml) were used to develop a calibration curve with a correlation coefficient of 0.994 when fit with a linear model. Eugenol concentration of the HB extract was 36 % (0.359 mg/ml).
The full scan of the HB extract detected appreciable amounts of eugenol, methyl eugenol, caryophyllene, camphor and naphthalene, with respective relative areas of 38, 25, 18, 9, and 4%. Each of those compounds was tentatively identified using NIST and Wiley MS libraries. Eugenol’s GC peak area identified during the full scan may have other components contributing to that area that are not eugenol, but may have like retention times. This phenomenon likely caused the slight overestimation between the full scan and the more accurate and sensitive SIM analysis done with a eugenol standard.

Discussion (4.6)

The significant reduction in GP between the EHB and HBOT samples is speculatively caused by one of two occurrences, or perhaps a combination of the two. The first being that the increased surface area (SA) that occurs when the 3-dimensional gel matrix is formed as the HB was encapsulated by the gel provided the ruminal microbes with more SA to adhere to, thus increasing organic matter (OM) degradation and fermentative GP. To illustrate the observable difference in SA following AI encapsulation, Figure 4-2 shows a photograph of the two samples of the same 4 % NaAlg gel emulsion, the first with HB encapsulated, and the second without.

![Figure 4-2](image-url). Photograph of 4-EHB (A) and 4-NHB (B) gels illustrating the observable difference in SA after holy basil has been encapsulated in a 3D gel matrix.
Increased SA has been linked to increased rates of microbial digestion and fermentative GP. By incubating meadow hay particles (size range of 0.1 – 2 mm) in rumen inoculum of Romney sheep fed the same hay, Gerson et al. (1988) determined that fermentative GP was more rapid as particle SA increased. When comparing larger particles (1 – 2 mm) with smaller ones (0.1 – 0.4 mm), the ruminal GP rate per m² SA was 450 % greater for the larger particles. The authors partially attributed this increased GP rate to an increased microbial population density, as the particles with a larger SA were shown to have a microbial population density approximately 600 % higher than the smaller SA particles (Gerson et al., 1988). Maaroufi et al. (2009) characterized the in vitro ruminal fermentation of various particle sizes of pea seeds and concluded that an increased specific SA contributed to an increased rate of particle fermentation. An increase in available SA accessible to ruminal microbes led to higher fermentative GP (Maaroufi et al., 2009).

Secondarily, it is speculated that HB and its secondary metabolites possess antimicrobial properties that moderate and effectively negate ruminal microbial action, ultimately reducing fermentative GP rates. This anti-microbial action may also be altering the VFA profile, effectively decreasing the acetate:propionate ratio which, if substantial enough, would induce a proportional shift in GP (Makkar, 2004). This concept would also help explain why so little fermentative gas was produced when incubating the samples of just dried and ground HB, and elucidate as to why there was such a significant difference in GP between the samples with HB added on top of the gel immediately prior to the incubation and the samples with no HB included at all (NBH).
As far as the EHB samples are concerned, the gel complex itself may have been microbially fermented and broken-down, leading to the high rates of GP, but the microcapsules safeguarding the HB particles may remain, for the most part, intact. If the HB remained encapsulated, and thus kept from direct contact with the microbial environment, the anti-microbial actions of the HB could not occur. This notion would lend evidence to the successfulness of the encapsulation process and would be supported by Templeman et al.’s results (Chapter 3) in which NaAlg and RBW gel complexes experienced significant DM disappearance rates (83 % average), though retained a high percentage of the encapsulated AI (81 % retention) throughout 48 hr in vitro rumen incubations.

A number of studies have demonstrated the anti-microbial/bacterial activity of HB against both Gram-positive and Gram-negative bacteria (Gupta et al., 2002; Viyoch et al., 2006; Chandrasekaran et al., 2012). Both volatile (leaves) and fixed (seeds) oil extracts of HB have been shown to exhibit strong anti-bacterial properties, inhibiting the growth and proliferation of a number of bacterial strains including *Staphylococcus aureus*, *Escherichia coli*, *Bacillus pumilus*, *Propionibacterium acnes* and *Pseudomonos aeruginosa* (Prakash & Gupta, 2005; Viyoch et al., 2006; Pattanayak, et al., 2010).

Eugenol and methyl-eugenol are believed to be the most influential in terms of HB’s anti-microbial effect, with the hydroxyl groups (–OH) attached to their respective aromatic rings being responsible for the anti-microbial activities (Farag et al., 2004; Viyoch et al., 2006; Soltan et al., 2007). The chemical make-up of HB can vary greatly between strains, or even within the same strain depending on the conditions of the environment (eg. planting season, time of harvest, geography, soil composition, etc.) in
which it was grown. In most cases however, including this study, eugenol and methyl-eugenol make-up large portions of the volatile oil (Mondal et al., 2009; Pattanayak et al., 2010; Sims et al., 2014). The 36 and 25 % eugenol and methyl-eugenol, respectively, identified in this study are comparable to past findings. Eugenol has been identified in HB extract in a variety of quantities including 40 % (Prakash & Gupta, 2005), 42 % (Viyoch et al., 2006), and 59 % (Trevisan et al., 2006). Methyl-eugenol has a similarly wide range of variation, being identified in quantities including 12 % (Viyoch et al., 2006), 20 % (Gupta et al., 2002), to upward of 78 % (Kothari et al., 2004).

Eugenol has been used as a natural agent to modify the microbial environment and fermentation process within the rumen. Soltan et al. (2011) studied the in vitro capability of carvacrol and eugenol to modify the rumen microbial fermentation and found that both additives, at varying concentrations, significantly reduced ruminal GP, as well as OM degradation and ruminal protozoa count, when compared to a control sample (no additive). Addition of 30 µl eugenol reduced total in vitro GP by 28 %, CH₄ production by 38 %, OM degradation by 18 %, and protozal count by 30 %. Castillejos et al. (2006) observed that eugenol altered VFA proportions in 24 h batch cultures, but without affecting the total VFA concentration. Eugenol at 5 mg/L reduced the proportion of acetate and the acetate:propionate ratio by 2 and 5 %, respectively (Castillejos et al., 2006).

**Conclusions (4.7)**

This study investigated the in vitro fermentative GP of HB encapsulated within NaAlg and RBW gel complexes and compared the results to GP rates of the individual
gel constituents. Of all the treatments analyzed, the EHB samples had highest rates of GP. When compared to the other treatments, the EHB samples produced, on average, 95 and 73% more fermentative gas at the asymptotic (maximal) and t24 GP levels, respectively. This significant difference in GP was speculatively attributed to either the larger SA of the encapsulated gels versus the same gel with no encapsulated AI, or to prevention of the anti-microbial actions of the HB. A GC-MS analysis of the essential oil extracted from HB identified considerable amounts of eugenol (36%) and methyl-eugenol (25%), the two constituents recognized as being the most influential anti-microbial compounds in HB’s essential oil. These findings support the potentially rumen-modifiable, anti-microbial actions of HB on the *in vitro* ruminal environment.

**Conflict of Interest (4.8)**

None declared.

**Acknowledgements (4.9)**

The authors are grateful for the financial support received from of the Dairy Farmers of Ontario (Mississauga, ON, Can) and the Ontario Centres Of Excellence (Toronto, ON, Can).
References (4.10)


Fermentrics™ Interpretation and Guidelines, 2013 http://www.fermentrics.com/guide.pdf (accessed 01.03.16)


Chapter 5.

SUMMARY AND CONCLUSIONS

In summary, this project led to the successful development of an organogel and NaAlg formulated gel complex that capably encapsulated dried and ground HB, and then investigated the ability of these gels to safeguard the HB from ruminal degradation. The first experiment determined that a RBW and canola oil based organogel represented the ideal option to represent the oil-based portion of the gel complex and proceeded to examine the DM disappearance rates of the gel via a 48 hr nylon-bag style *in vitro* incubation. It was determined that, when compared to an unprotected AI, the gel complex (most notably the 4% NaAlg based gel) significantly reduced the loss of the encapsulated AI.

The second experiment investigated the fermentative GP resulting from *in vitro* 48 hr incubation of the gel complexes as well as the individual constituents that comprises the gels. While gels with encapsulated HB in fact produced the most fermentative gases, the data provided unusual results that required further investigation. The most notable of those included the significant reduction in GP between encapsulated gels and those with the same amount of HB (AI) added on top of the gel, and not encapsulated within. This significant difference was speculatively attributed to either the larger SA of the encapsulated gels versus the same gels with no encapsulated AI, or to prevention of the anti-microbial actions of the HB *in vitro*, or perhaps a combination of these two occurrences. The potentially rumen-modifiable anti-microbial actions of HB led to an extraction of HB’s essential oil and the determination of the oil’s chemical
profile via a combined GC-MS analysis. The considerable amounts of both eugenol (36 %) and methyl-eugenol (25 %) identified support the anti-microbial effects the HB potentially had on the ruminal environment, as those constituents are recognized as being the most influential anti-microbial compounds in the essential oil of HB.

For future studies, it would be worthwhile to investigate the gel complex’s capacity to encapsulate and safeguard hydrophobic AIs in order to determine if the gels are more capable of encasing and rumen-protecting fat-soluble rather than water-soluble AIs. As well, it would interesting to examine the potential of these gels to deliver and rumen-protect AIs that are not intended for expression in milk, but instead are for manipulation of enteric absorptive capabilities or are anticipated to modify or improve the intestinal environment, and need to remain intact through the rumen for these purposes. Lastly, it would be of interest to further explore the anti-microbial and rumen-modifiable abilities of HB and its active secondary metabolites, as well as the exact mechanisms in which HB exerts its anti-microbial actions. With the limited body of literature exploring HB as a natural modifier of the ruminal environment and VFA profile, it would be valuable to look into the effects of HB, or specific constituents of HB, on the gut health and milk production of dairy cows.