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Novel Protected Gelatin Capsules Containing Fish Oil Mitigated the Effect of Milk Fat Depression and Reduced Rumen Degradation Compared to Untreated Capsules

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NOVEL PROTECTED GELATIN CAPSULES CONTAINING FISH OIL MITIGATED THE EFFECT OF MILK FAT DEPRESSION AND REDUCED RUMEN DEGRADATION COMPARED TO UNTREATED CAPSULES

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ABSTRACT

The objective of this study was to assess the effects of feeding gelatin capsules treated with alcoholic solutions of flavoring agents followed by drying containing fish oil on lactation performance, rumen fatty acids content and milk enrichment of fatty acids. In Trial 1, four multiparous ruminally-fistulated Holstein cows were randomly assigned to one of four dietary treatment sequences in a 4 x 4 Latin square design. Treatments consisted of 1) Control with no capsules, 2) Control plus 200 untreated capsules per cow/day, mixed with the TMR; 3) Control plus 200 treated capsules per cow/day placed directly into the rumen, 4) Control plus 200 treated capsules per cow/day, mixed with the TMR. The total dose in treated and untreated capsules was 28 g of EPA and 13 g of DHA. In Trial 2, three fistulated Holstein and three fistulated Jersey multiparous cows were randomly assigned to three dietary treatment sequences in a replicated 3 x 3 Latin square design. Treatments consisted of 1) Control with no capsules fed to the cows, 2) Control plus 180 untreated capsules per cow/day, and 3) Control plus 180 treated capsules per cow/day. The total dose in treated and untreated capsules was 15.58 g of EPA and 12.75 g of DHA. Compared to control, feeding fish oil capsules significantly (Trial 1) or numerically (Trial 2) reduced milk fat concentration and yield. Furthermore, feeding untreated or treated capsules in both trials did not affect animal performance or milk composition. Compared to controls, supplementing the diet with fish oil capsules in both trials consistently increased total *trans*-C18:1 isomers and DHA concentration in the rumen and milk fat. However, for both trials, capsule protection treatment had a minimal effect on the concentration of any of the reported rumen and milk fatty acids. When assessed under laboratory control conditions,

the treated capsule weight was increased by 40% due to water absorption, while resistance to pressure decreased by 84% after 2 hours of incubation in water. Results of this study suggest that due to reduced capsule shell resistance to abrasion, treated capsules marginally prevented the release of fish oil in the rumen.

Keywords: rumen protection, EPA and DHA, milk fat

DEDICATION

In memory of my cousin Raul Andres Peña Poveda, his advice, love, and smile will be always in my heart.

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TABLE OF CONTENTS

Page

Table of Contents (Continued)

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER ONE

REVIEW OF LITERATURE

Introduction

Fat is a crucial component of milk, to the point that it defines the pricing system of most milk marketing orders in the US (Heinrichs et al., 2016). Knowing the fatty acid profile of milk could be very important worldwide, particularly in developed countries, where fat from cattle sources is often the primary energy source in human diets (Sanders, 2016).

The consumption of fat is linked to adverse effects in humans, it may cause disorders such as cancer and coronary heart disease (Giovannucci et al., 1993; Williams, 2000). High-fat consumption can elevate cholesterol levels in the blood (Hegsted et al., 1965), sticking to the walls of the arteries, decreasing their elasticity and limiting the normal flow of blood through the body (Salter, 2013). However, depending on its specific fatty acid (FA) profile, fat consumption may positively influence immune function and human health (Calder, 2008). For instance, conjugated linoleic acid (CLA), mainly obtained from ruminant-derived products, has proved to be anti-atherogenic, antidiabetogenic, anti-obesity, immunomodulation, and modulator of bone growth (Belury, 2002). Moreover, yogurt and other fermented forms of milk have shown therapeutic benefits on some digestive disorders, such as recolonization of the intestine post-antibiotic treatment, gastric acidity, gastroenteritis, and flatulence, among others (Gorbach, 1990).

For this reason, changes in human nutrition have been strongly recommended in recent years, including changes in the intake ratio of polyunsaturated fatty acids (PUFA)

to saturated fatty acids (Wood et al., 2004). The amount of saturated fat should not exceed 10% of the total diet (Williams, 2000). However, not all saturated FA is bad for us; some of them, such as stearic acid, do not increase total cholesterol or pressure in the blood (Zock et al., 1995).

Milk and its subproducts are known for being a vital source not only of energy but also high-quality protein, vitamins and minerals (Lock et al., 2004). Fatty acids contained in milk could play a role in contributing to the prevention of many chronic diseases. For instance, omega-3 (n-3) fatty acids have been considered beneficial. Studies demonstrated that polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are antiatherogenic, antithrombotic, anti-inflammatory, and immunosuppressive (Ip et al., 1999).

The n-3 fatty acids are present in our diets at low levels due to our low consumption of fish and seafood (Williams, 2000). Therefore, their supplementation in dairy cattle diets may produce positive health benefits for both the animal and the consumer (Medeiros et al., 2007). However, the precursor of n-3 in grasses (Linolenic Acid) is extensively biohydrogenated by rumen bacteria (Kitessa et al., 2004). Thus, feeding rumen-protected fat (RPF) in dairy cattle is one of the strategies to reduce incomplete biohydrogenation, contributing to the energy supply of the cow without causing adverse effects on fermentation and increasing the content of omega 3 FA in milk (Palmquist et al., 1980).

This chapter will first review the importance of using fat in ruminant diets, its effects and behavior in the rumen of the cow and its post-ruminal digestion. The second part is the technology available to protect fat and its implications in ruminal nutrition.

Finally, a little overview of protected fat efficiency and the factors that affect their degradation in the rumen as some critical studies done with some of those methods.

Fat Utilization in Dairy Diets

Fats are a group of compounds that are not commonly soluble in water but in ether or other organic solvents. This characteristic is given by the lack of a polar group where water could be attached. They can be found to be oils or fats depending on their physical state, provided by temperature. Fat is mainly found in solid texture, while oils are usually in more liquid forms. Depending on the degree of saturation, fat's melting point will change. More saturated fats are commonly closer to solid states at room temperature. At the same time, fat is composed of individual FA, which are carbon chains linked to hydrogen and oxygen atoms usually attached to a glycerol backbone. The characteristics of those FAs will determine fat's nutritional value and other physical properties.

In animal feed, fats are commonly bound to glycerol groups. In forages, fat is mainly present in glycolipids, while in cereal grains like corn, the main form is triglycerides, three individual FAs attached to a glycerol molecule. That is also the primary form of lipid stored in animal tissues and the main type of fat in milk (98%) (MacGibbon et al., 2009).

In the dairy industry, fat has evolved from a contaminant in some protein dietary supplements to a high and valuable energy source (Palmquist et al., 2017). Grazing ruminants typically consume limited amounts of fat (Hess et al., 2008). Thus, fat is often offered as a supplement to meet their energy requirements due to its higher caloric density

value (Doreau et al., 1997). Fat supplementation has increased mainly in high-producing herds due to their higher energy demands. (Moallem, 2018). However, fat can also be used to manipulate the absorption and digestion of other nutrients in the diet and to enhance the reduction of greenhouse gas emissions at the farm level. For instance, using extruded linseed reduced linearly CH_4 emissions when it was supplemented at 5, 10, and 15% of the dietary DM without affecting intake, digestibility, or milk production in multiparous Holstein cows in both hay and corn silage-based diets (Martin et al., 2016). In addition, lipid intake can affect the proportion of specific fatty acids in milk and meat, allowing us to manipulate the lipid content of this product (Chilliard, 1993).

However, a high increase in the proportion of lipids can also negatively affect ruminal fermentation and digestibility (Jenkins, 1993). Depending on the amount of fat fed, the digestion of structural carbohydrates can be reduced, impacting the production of hydrogen, methane, and volatile fatty acids (VFAs) in the rumen (Ikwuegbu et al., 1982).

Typically, in a grass-based diet, the lipid content is up to 3.0% of the dietary dry matter (DM) (Dhiman et al., 2005), and this percentage should not exceed 6 to 7% (DM basis) even in high-producing cows to minimize the impact on rumen fermentation (Jenkins, 1993; Van Soest, 1994). Primary dietary fat sources are oilseeds like soybean, corn and cottonseed, canola, rapeseed, flaxseed, safflower, and sunflower (Moallem, 2018). All those ingredients have a common characteristic: their primary type of fat is unsaturated and polyunsaturated (Moallem, 2018), meaning that most of the FA contained in them has one or more double bonds.

Effects of dietary fat on milk production and composition

The nutrient composition of milk is not static. It varies according to the state of lactation, feeding, the health status of the animal, genetics (Fox, 2009) and the combinations of all those factors. Moreover, depending on geographical location, each region's different practices and diets are translated into different compositions and nutritional values. However, changes in nutrients such as protein are very modest (Jenkins et al., 2006) compared to the fatty acid profile and proportion of milk, which is often the most affected (Heck et al., 2009).

Due to its FA diversity, ruminant milk is unique among all terrestrial mammals (Palmquist, 2006). Jensen (2002) reported 416 different FAs found in bovine milk lipids. Dietary fat (Beaulieu et al., 1995) and rumen fat metabolism (Jenkins et al., 2008) affect milk FA composition. In addition, feeding supplemental fat may enhance lactation performance (Grummer, 1991). According to Palmquist (1993), other factors like animal genetics, stage of lactation, and de novo synthesis may affect milk's lipid composition, being the diet the factor that would change milk fat composition the most. It also influences urea, protein, calcium, and flavor. This change would be primarily linked to the triglyceride fraction, which represents approximately 95% of the total milk fat.

As early as 1966, studies have evaluated the effect of adding different amounts of fat to the diets of dairy cows. Some results indicated that milk yield was increased by 41% and fat content by 56% when passing from 37 to 129 g/d of fat added to the diet. Another study (Banks et al., 1976) showed that increasing the amount of fat by almost 500% (from 81 to 481 g/d) caused an increase in milk yield of 36% and 55% of milk fat yield. Thus,

research was needed to set a certain percentage of fat in the total diet of a dairy cow to reach the maximum potential milk yield (Clapperton et al., 1983).

Several studies (Emmanuel et al., 1984; Mandell et al., 1996; Keady et al., 2000; Chilliard et al., 2001; Sanz et al., 2002; McNamara et al., 2003; Chow et al., 2004; Perfield et al., 2004; Shingfield et al., 2009), have been conducted on sheep and beef cattle to understand the effect of supplemental fat utilization. For instance, one showed that when increasing fat intake in Holstein and Jersey cows to 0.75 kg/d, DMI decreases, but it does not affect milk yield, protein, or fat content (Beaulieu et al., 1995). Commonly, the fat intake of cows on forage-based diets is as low as 2% of the DM, and including one more percentual unit is recommended to obtain a higher benefit from energy and other dietary components of those forages. Conversely, high-producing animals are offered higher amounts of energy in their diets to fulfill their requirements. In this case, including fat can be up to around 6% of the dietary DM to prevent adverse effects of rumen metabolism (Hess et al., 2008).

Energy intake affects milk fat composition in many ways. If the energetic balance of the cow is positive, dietary carbohydrates and fat influence the FA composition of milk. High carbohydrate intake decreases the milk concentration of short-chain FA, and C18 is more likely to increase depending on the source and its fat content. Cows can synthesize FA through a process named “De novo synthesis,” which transforms FFA into C4:0 to C14:0 (short-chain FA) and derives half of the C16:0 FA in milk. The primary source of carbons used for this synthesis come from Acetate and 3-OH-butyrate. The other part of C16:0 and the rest of the long-chain FA come from circulating blood lipids, the second

major FA source (Grummer, 1991). Thus, the FA going to the duodenum comes from dietary and microbial origins.

When the cow goes through a negative energy balance, the supply of acetate and glucose decreases, depressing the synthesis of short-chain FA in the mammary gland, which at the time causes a higher mobilization of adipose tissue (Palmquist et al., 1993).

It was thought (Grummer, 1991) that the fat composition in milk should be close to these values: 8% saturated FA, 10% polyunsaturated FA, and 82% monosaturated FA. However, those values are difficult to achieve due to the ruminal BH of FA. Biohydrogenation is the process where microorganisms extensively hydrolyze dietary lipids in the rumen, producing a high concentration of saturated FA and a lower concentration of polyunsaturated FA, particularly omega-3 FA in milk (Simopoulos, 2002), which is highly hydrogenated.

Moreover, the incomplete BH of dietary linoleic acid results in cis-9 trans-11 Conjugated Linoleic Fatty acid (CLA), representing more than 82% of CLA in dairy products. In 1999, it was demonstrated that this FA reduced mammary tumor incidence in rats when added to their diet (Ip et al., 1999). One way to increase its concentration in milk fat is by feeding fish oil, extruded soybeans, or their combination (AbuGhazaleh et al., 2002). Although CLA supplementation reduces the body fat content of growing animals, when fed more than 2%, a higher supplementation can reduce milk fat synthesis in dairy cows (Baumgard et al., 2001). On the other hand, positive effects have been observed in milk fat when CLA is supplemented since they are naturally part of ruminant feed.

The supplementation of fat also affects the protein proportion of milk. On average, every 100 grams of supplemental fat intake can cause a depression in protein content of 0.1 to 0.3% (Jenkins et al., 2006).

Effect of breed on milk fat composition.

Even before the evidence of many studies, in the middle 90s, the differences in milk fat content among breeds started to be evident. For instance, Krukovsky (1961) reported that Holsteins had higher iodine value than Jerseys (DePeters et al., 1995), suggesting that the content of unsaturated FA in milk fat was influenced by breed. The percentages of C16:0, C16:1, and C18:1 were higher in Holsteins compared to Jerseys (Stull, 1964). Varman (1968) tested four different breeds to see the changes in blood FA in response to specific treatments. They measured plasma levels of phospholipids, cholesterol, esters, free cholesterol, free FA, and acetate. They also measured changes in milk fat percentages, finding significant differences driven by breed (Varman et al., 1968).

Karijord et al. (1982) evaluated the effect of dairy cow breeds on milk yield and milk FA composition. The authors observed genetic variation within breeds and indicated that a higher heritability effect could be observed depending on the size of the sample population. A small sample size could diminish any breed effect. However, it increases considerably if the averages of several samples are used (Karijord et al.,1982). The same authors also found a genetic correlation between the proportion of short-chain FA (C6:0 to C14 0) and the percentages of fat and protein.

Beaulieu (1994) reported differences in the proportions of FA in milk from Holsteins and Jerseys during weeks 7 to 14 of lactation under the same dietary treatments. The author conducted an experiment using four different levels of inclusion of calcium salts of palm fatty acid distillate (0.25, 0.50, 0.75, kg/d) to test milk yield and milk fat composition. Dry matter intake decreased with higher levels of dietary fat inclusion, but milk yield, protein, and fat content were not affected. The proportions of FA in milk were altered by the level of fat inclusion in the diet compared to a control treatment.

Jersey cow milk contained a higher proportion of short-chain FA (SCFA) and medium-chain FA (MCFA), with a lower proportion of palmitic and oleic acid than Holstein milk fat. Arnould (2009) explains this phenomenon not only for Jersey cattle but also for other breeds, such as Montbeliarde and Normande, by the action of Delta-9 desaturase (SCD), which is the enzyme in charge of introducing a *cis*-double bond between the carbons 9 and 10 of 10 to 18 carbon chain-length saturated fatty acids. Thus, the activity of SCD is commonly higher in Holstein cows compared to Jersey ones.

The addition of dietary fat inhibits the de novo synthesis of milk FA. The longer the FA chain, the higher the inhibition is compared to the control (Figures 1.1 and 1.2). Nevertheless, other studies found no differences in C18:2 among breeds when fed different fat levels (DePeters et al., 1995). Finally, other authors (Soyeurt et al., 2006) suggest that the differences between Holstein and Jersey milk FA profiles are significant, except for palmitoleic acid C16:1, which remains similar in both breeds, as shown in Table 1.1. This table also compares Holstein milk fat and fatty acids with the other three breeds (Dual Purpose Belgian Blue, Meuse-Rhine-Yssel type Red and White, and Montpeliarde).

Characterization of Unsaturated Fatty Acids

Unsaturated FAs are those FAs that contain one or more double bonds on their chain. The number of double bonds they have will determine whether they are monounsaturated or polyunsaturated. These bonds are rigid structures, and the molecules that have them can exist in two isometric forms, *cis* and *trans*; depending on the position of their chemical groups, they can be on the same side or the opposite. Oleic acid (18:1) and linoleic acid (18:2) are living organisms' most abundant monounsaturated and polyunsaturated FA. Naturally, fats and oils usually contain only *cis* fatty acids (Michael et al., 2016), and *trans* fatty acids are more often the result of manufacturing processes such as adding hydrogen to vegetable oil.

Trans fatty acids originate from either the biohydrogenation pathway of dietary unsaturated fatty acids by rumen bacteria or chemical processing (partial hydrogenation) of unsaturated fatty acids (e.g., vegetable oils). In natural products, the predominant *trans* isomer is vaccenic acid (*trans*-11 C18:1) (Donald et al., 2005), while in industrial production, the predominant *trans* isomer is elaidic acid (*trans*-9 C18:1).

A characteristic of the UFA is the lower melting point compared to saturated FA due to a lower requirement of energy to disrupt the intermolecular forces. Thus, the more unsaturated the fatty acid is in a fat/oil source, the ‘softer’ the oil is. Also, the more double bonds there are, the more susceptible to oxidation. This phenomenon includes the tendency of oils to become rancid (McKee et al., 2012).

In addition, UFA is classified and named according to the location of the first double bond relative to the molecule's terminal methyl (omega, ω) end. The sequences of

double bonds in a molecule are always three carbons apart. Omegas 3 and 6 are unique FA due to the position of double bonds in the chain. In addition, mammals cannot synthesize them and must be supplied in the diet (Meyer et al., 2003). Several vegetable oils contain a high proportion of omega-6 fatty acids. Still, there are only a few sources of omega-3 in nature, the most common being grasses, linseed, and fish oil/marine sources. Omega-3 and omega-6 fatty acids are very active in the body, stimulating aspects of the immune system (Omega-6) and anti-inflammatory responses (Omega-3). When Omega 3 FA comes from marine products, its concentration depends on the season and geography of the product. Those characteristics could generate a variation in the concentration from 4 to 32% (Chilliard et al., 2001). However, consuming these fatty acids is deficient in most Western diets (Rego et al., 2005).

In dairy cattle, both n-3 and n-6 FA should be supplied in the diet since they cannot synthesize them. However, feedstuffs contain high amounts of n-6 FA, while n-3 input is almost limited to flaxseed and fish oil (Moallem, 2018). Several authors (Wonsil 1994; Donovan 2000; Rego 2005) have supplemented this FA and reported a decrease in milk production when it was added to the diet at a rate of 3% of diet DM. According to Rego (2005), protein content and protein yield can be reduced due to higher concentrations of fish oil in the diet. They also found a negative correlation between milk fat content and concentration of milk fat of *trans-C18:1* FA, CLA's, EPA, and DHA. Finally, they found a positive relationship between C18:0 and *cis*-9 C18:1 and between *trans-C18:1* and CLA in milk fat.

Omega-6 fatty acids include γ-linolenic acid (GLA), arachidonic acid (AA), and linoleic acid (LA) (Jalč et al., 2018), which is the principal polyunsaturated fatty acid in the western diets (LA; 18:2 n-6). Since humans cannot synthesize it, it is known as an essential FA and can be found in vegetable oils, nuts, and seeds. Being supplied, Linoleic acid can be used to obtain other polyunsaturated fatty acids through elongation and desaturation processes. Omega-6 FA is part of the cell membranes' structure involved in the inflammation response processes (Innes et al., 2018). Gamma-linolenic acid has shown positive responses to different types of cancer, such as breast, pancreas, colon, and brain cancer, inhibiting a protein involved in cell migration and reducing tumors. The intake of this FA also improves the efficacy of the anticancer drugs (Kapoor et al., 2006).

DHA and EPA are synthesized from the n-3 alpha-linolenic acid (ALA, 18:3n-3) and n-6 linoleic acid (LA, C18:2n-6) through desaturation and elongation processes. Since animals cannot synthesize LA and ALA, they must consume it in their diet. There is enough evidence that in ruminants, the amount of n-3 in plasma, red blood cells, meat, milk, and reproductive tissue is related to the concentrations of those fatty acids in the diet (Gulliver et al., 2012). There are many sources of ALA and LA in ruminant diets, such as forages, linseed, grains, soybean, safflower, sunflower, fish oil, and fishmeal. Other sources come from some species of algae used to produce ethanol and biodiesel. (Gulati et al., 2003)

Rumen Metabolism of Fat

Due to rumen microorganisms and their interaction with the feed, the nutrients that come into the diet are altered in this chamber. Thus, they differ from those

that leave the rumen after fermentation and reach the intestine, where they will be absorbed and used for milk synthesis (Doreau et al., 1997). After studies of ruminal lipid metabolism, two significant microbial transformations were identified in the rumen: lipolysis and biohydrogenation (Jenkins, 1993). Due to those processes, the absorption of FA in a cow's intestine is very low and depends on the formation of bile salt micelles and their size. A micelle is an aggregate of amphiphilic molecules that can transport fat through the organism due to their hydrophilic surface and hydrophobic center. The bigger the micelle, the higher the fat can carry and the easier the digestibility will be (Zinn et al., 1999).

Lipolysis

Once dietary fat enters the rumen, the first step in fat digestion is the hydrolysis of the ester linkages found in triglycerides, phospholipids, and glycolipids. This process is mainly performed by two bacteria species, *Anaerovibrio lipolytica,* which hydrolyzes triglycerides, and *Buturivibrio fibrisolvens*, which hydrolyzes phospholipids and glycolipids. However, there is also evidence of a significant role of rumen protozoa and fungi in this first process (Bauman et al., 2003). Lipolysis is the separation of the lipids releasing FFA, glycerol and sugars. The sugars and glycerol coming from the glycolipids are fermented into volatile fatty acids. This breakdown occurs when the dietary lipids are exposed to bacterial fermentation in the rumen. The unsaturated free fatty acids released are extensively biohydrogenated and converted into more saturated forms of fatty acids.

Biohydrogenation

Biohydrogenation is the process in which ruminal bacteria convert unsaturated fatty acids (USFAs) to saturated fatty acids (SFAs). Hence, the fatty acids leaving the rumen are highly saturated (Jenkins et al., 2008). Biohydrogenation is the second major transformation of dietary lipids in the rumen (Harfoot, 1981). Unsaturated FAs are more toxic than SFA to rumen bacteria, mainly to the ones involved in fiber digestion, who use BH as a detoxification reaction. Moreover, bacteria that produce hydrogen, a methane precursor, are inhibited by fatty acids, so BH is an alternative method to reduce equivalent disposal (Russell, 2002). It was first observed in 1951 and explained in detail later when Reiser incubated linseed oil in the rumen of sheep and realized that linoleic acid content in linseed oil decreased 25% after incubation while the content of saturated FA increased. This (Reiser et al., 1956). Further studies realized that the end product of the BH process was stearic acid, passing through C18:1 and all the intermediates (Figure 1.3) (Jenkins et al., 2008).

We still do not know everything about the role of microorganisms involved in rumen BH, but bacteria and protozoa are the main ones involved in it. The presence of protozoa is unnecessary for BH to occur, although protozoa lipids are known for having proportionally more PUFA. They could represent an essential source of this FA for incorporation in milk and meat. The process of BH involves an isomerization of the double bonds, followed by their hydrogenation that occurs until the production of stearic acid (18:0).

Two types of microbial enzymes are involved in this 2-step process: isomerases and reductases. *Butyrivibrio fibrisolvens* were identified as the most important bacteria involved in the isomerization of FA that, in the case of linoleic acid, result in the formation of *c*9, *t*11-CLA, *t*10, *c*12-CLA, *t*10, *t*12-CLA; *t*9, *t*11-CLA; and *t*8, *t*10-Conjugated Linoleic Acid (CLA), among others. Conjugated linoleic acid is a term for octadecadienoic acid isomers. The double bond can be located anywhere from the carbon 6^{th} to the 16^{th} position. Following our example, the second step could be the hydrogenation of *cis*-9, *trans*-11-CLA to *trans*-11 18:1, where *C. proteoclasticum* is the main microorganism involved and the only bacteria that produces 18:0. Despite its name, these bacteria's action is deeply connected to *B. fibriosolvens* (Palmquist et al., 1980; Jenkins et al., 2008).

For a long time, it was believed that the bacteria in charge of BH could be divided into two different populations. Population "A" converts C18:2 to C18:1 but not significantly to stearic acid, and population "B" converts C:18:1 into stearic acid. Group A is more active than Group B. White et al. (1970) stated that Gram-negative capsulated anaerobic bacillus could hydrogenate 80% of unsaturated fatty acids in 72 hours. More recent studies (Lourenço et al., 2010) based on the taxonomy of the microbes suggest that a single type of bacteria can convert linoleic acid into stearic acid. However, this is still not completely clear.

Compared to linoleic acid, the biohydrogenation of linolenic acid is less understood. However, it was found by Kepler et al. (1967) that *B. fibrisolvens* act in similar ways in the biohydrogenation of both fatty acids. As a precursor of DHA and EPA (Figure

1.4), linolenic acid is also biohydrogenated in the rumen to stearic acid. It could leave intermediates such as trieonic, dieonic and monoeonic *trans-FA* (Lee et al., 2011).

The BH of USFA to SFA is not an extensive process and could result in the production of several unique fatty acids as biohydrogenation intermediaries, some of which will leave the rumen and be taken up in milk fat. Nonetheless, some of the intermediates of *trans* fatty acids formed in this process can cause a significant reduction in milk fat production.

Post-Ruminal Digestion of Fat

The fats that reach the abomasum are mainly free FA attached to feed particles and phospholipids from the microbial cell membranes. These FAs are predominantly saturated and become part of micelles made of bile salts and pancreatic secretions that will be absorbed on the surface of intestinal cells (Palmquist et al., 1980; Drackley, 2013). In general, fatty acid digestibility in ruminants decreases with degrees of saturation. The absorption process, which occurs predominantly in the jejune and the small intestine, is possible because of a compound called lysolecithin, a very efficient free FA emulsifier. In addition, individual fatty acids such as oleic acid can also help form micelles and improve fat digestibility due to their ability to function as amphiphilic agents (Lock et al., 2006). Once absorbed by the intestinal cell walls, free FA are converted back to triglycerides by using glycerol from blood glucose metabolism and then packed into chylomicrons and lipoproteins before entering the lymphatic system and be delivered to tissues where they can be used as an energy source, for milk fat production, or body fat deposition

Unsaturated FA are more digestible than saturated (Figure 1.5). The digestibility of oleic acid (C18:1), linoleic acid (C 18:2), linolenic acid (C 18:3), palmitic acid (C 16:0), and stearic acid (C 18:0) are 80, 78, 77, 75 and 72%, respectively. In the duodenum, the amount of the same fatty acid might influence the digestibility of it. For instance, C 18:0 digestibility decreases when its concentration increases (Boerman et al., 2015)

Flow of microbial lipids

Rumen bacteria are mainly made of triglycerides and phospholipids, such as phosphatidylethanolamine and phosphatidylserine. This group of bacteria represents around 10% of their dry weight (Domingues et al., 2016). The phospholipids of the ruminal bacteria are not as complex as those of protozoa. Modification of lipids by rumen microorganisms is related to the formation of many odd-carbon and branched-chain acids due to the incorporation of propionyl, 2-methylbutyryl, and 3-methylbutyryl moieties into the carbon skeletons. Microbial lipids are the result of the synthesis and modification of dietary lipids. The 15-carbon linear and branched acid chains represent the main components (Van Soest, 1963). Due to the lower pH in the abomasum, all bacteria and protozoa disintegrate, releasing their FA, which facilitates digestion in the gastrointestinal tract. Microbial fat that reaches the small intestine accounts for up to 15% (Drackley, 2013)

Effect of Milk Fat Inhibitors on Milk Fat Synthesis

Several theories have been proposed to explain the cause of milk fat depression (MFD). One of them suggested that the main reason for MFD was an inadequate supply of

lipid precursors for mammary gland synthesis of milk, such as deficiency of acetate or ß-hydroxybutyrate. Still, the effect of both was denied after research (Shingfield et al., 2007). However, Urrutia (2017) found that increasing the supply of acetate increased milk fat synthesis of dairy cows, mainly the 16-carbon-chain FA fraction. This effect occurred only on the last day of treatment supplementation. Thus, further investigation is required to fully understand the role of acetate in milk fat synthesis.

Another theory talked about inhibiting one or more steps in the milk fat synthesis process in the mammary gland (Baumgard et al., 2002). Grant and collaborators reported that the particle size of the feed could affect milk fat synthesis. For instance, depending on this characteristic, silage fed to the cows could affect milk fat percentage; fine silage particles would decrease its percentage from 3.8 to 3.0%, even though milk yield would not decrease (Grant et al., 1990).

A glucogenic theory for MFD indicates that feeding high levels of starch would increase the propionate proportion produced in the rumen, enhancing hepatic gluconeogenesis and increasing plasma glucose. This will stimulate insulin release, causing a suppression in the release of FFA (Emmanuel et al., 1984). An alternative theory proposed that feeding higher fat levels would produce a physical coating of the nutrients or the microbes, reducing ruminal fermentation. As a result of the lower fermentation, less acetic acid would be released to the mammary gland, resulting in a lack of energy for milk fat synthesis (Staples, 2006).

Diet-induced milk fat depression could occur when there is an alteration in rumen fermentation, for instance, a change in pH, or diets containing high levels of unsaturated

fatty acids, affecting the normal biohydrogenation pathway and resulting in more intermediates. This is also called the 'biohydrogenation theory' of milk fat depression. In this theory, the intermediates of the fatty acid biohydrogenation pathway exit the rumen, where upon reaching the mammary gland, they can decrease the expression of lipogenic enzymes and, subsequently, milk fat production.

The major fatty acid identified as being involved in milk fat depression is *trans*-10, *cis*-12 (CLA), *trans*-9, *cis*-11 CLA, and *cis*-10; trans-12 CLA are also milk fat inhibitors. The *trans*-10 *cis*-12 CLA isomer formed as an intermediate of rumen BH is a potent inhibitor of milk fat synthesis and an MFD inductor in dairy cows. Baumgard et al. (2001) infused different doses of *trans*-10 *cis*-12 abomasally. They found a relationship between the percent reduction in milk fat yield and the amount of *trans*-10, *cis*-12 CLA and the milk fat content of *trans*-10, *cis*-12 (Baumgard et al., 2001; De Veth et al., 2004).

Because of the ruminal microbe's action (mainly under rumen acidic conditions), this FA can be converted to *trans*-10 C18:1 (Bauman et al., 2003). There is no evidence that the mammary gland can synthesize *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA. Thus, those FAs leave the rumen and reach the small intestine, where they are absorbed into the blood and transported to the mammary gland, where they are incorporated into milk fat. According to Bauman (2003), this FA reduces the amounts of mRNA for lipogenic genes in tissue, partially inhibiting the action of the enzymes in charge of the synthesis of short and medium-chain fatty acids by the mammary gland.

However, as mentioned earlier, *trans*-10 *cis*-12 CLA does not explain all the causes of MFD, and that is why other fatty acids have been tested as potential inhibitors of milk

fat synthesis (Shingfield et al., 2007). Porterfield et al. (2007) tested the effect of infusing abomasally a mixture of 9, 11 CLA (*trans*-9, *trans*-11, *trans*-9, *cis*-11, *cis* -9, *trans*-1) and found that this mixture, compared with the control, reduced milk fat yield by 15%.

Harvatine et al. (2009) conducted a study to demonstrate the effect of sterol response element-binding protein 1 (SREBP1) on milk fat synthesis regulation. They showed an essential role as a signaling pathway for the transcription of lipid synthesis genes in the mammary gland when milk fat depression occurs. However, this phenomenon is still a matter of research.

Technology Available to Protect Fatty Acids from Rumen Metabolism.

Due to the adverse effect on the rumen bacteria, fiber digestion and rumen function of supplementing high levels of lipids, several approaches (encapsulation, extrusion, and calcium salts of FA) have been developed to protect fats from the rumen environment. Rumen-protected sources of fat are a tool that helps to avoid the adverse effects because they are not or almost not affected by the rumen environment, leading fat to be released later in the small intestine for digestion, absorption, and ultimately incorporation into milk fat (Grummer, 1991; Moallem, 2018).

The goal of increasing the energy supply to dairy cows without adverse effects on ruminal fermentation (Palmquist et al., 1980) can be accomplished by controlling the levels of unsaturated fatty acids available in the rumen. Thus, density and particle size are critical to determining the escape of protected feed from the rumen. Fatty acids that stay too long

in the rumen may cause lowered milk fat, but the host animal may use the ones that escape the rumen to increase unsaturated FA in tissues and milk (Van Soest, 1963).

The type of protection that has been widely used is encapsulation. However, there are many ways to achieve encapsulation, such as protecting fatty acids surrounded by proteins that cannot be degraded by crosslinking them with formaldehyde. Also, by encasing nutrients within a sphere of high melting point saturated fatty acids (Jenkins et al., 2007). Blocking the carboxyl group is another method to protect fatty acids from hydrogenation since it is required for bacteria isomerase to start eliminating double bonds. This blocking can be accomplished by forming an ionic bond with calcium or forming fatty acyl amides. These calcium salts and the fatty amide are not absorbed in the rumen. Postruminally, fatty acids are released in the abomasum mainly by acidic dissociation, while fatty amides release the unsaturated fatty acids in the proximal duodenum (Jenkins et al., 2007). However, feeding protected fats can lead to protein imbalances. Energy from fat dilutes energy from carbohydrates, which is the primary source of energy for rumen microorganisms. Feeding high amounts of protected fat could require feeding increased amounts of protected protein (Van Soest, 1963).

Calcium salts

Calcium salts are formed by creating an ionic bond between the carboxyl group of the fatty acids and Ca ions (figure 1.6). Fatty acids can be treated with calcium to generate a complex that is not soluble in the rumen and protects the microorganisms from excessive amounts of fat (Russell, 2002). The most common are based on palm oil fatty acids, which

are stable in the rumen at a normal pH (pH above 6.0), so they are inert to fermentation. The dissociation occurs later in the abomasum's acidic conditions, and both FFA and Ca ions are available for absorption in the small intestine (Square, 1988). Both physical characteristics and the type of fatty acids used in their manufacture can significantly affect the stability and function of the Ca salts. For instance, the duodenal flow of 18:2 was numerically higher for calcium salts of palm fatty acids compared to calcium salts of rapeseed fatty acids. Still, there was no statistical difference (22.7 vs. 17.1 g/d). However, C18:2 produced in milk was higher when Ca salts of rapeseed fatty acids were used (23 vs. 27 g/kg total FA; Enjalbert et al., 1997), and according to Lundy (2004), calcium salts protection worked similarly than converting polyunsaturated fat to amides (see following protection method). Both methods allowed an increase of linoleic acid to the duodenum at a rate of 25 to 39 g/day. A possible side effect was described by Lohrenz (2010), concluding that when calcium salts were fed, DMI was lower compared to a control treatment; however, energy and protein intake did not decrease, and milk yield increased.

Fatty acyl amide

This type of protection relies on an amide bound of the UFA with an amine (figure 1.7). Fotouhi (1992) reported that amides were resistant to biohydrogenation and caused less harm to rumen fermentation compared to a control. The method was initially created to bypass methionine through the rumen linked to the carboxyl end of stearic acid. Still, the amide bond resisted ruminal biohydrogenation and started to be used to protect unsaturated fatty acids (Fotouhi et al., 1992). Like Ca salts, the preparation of amide

protection requires free FA as a precursor. According to several published studies, its performance in the rumen is also very variable (Reeves et al.,1998; Lundy et al., 2004; Perfield et al., 2004). For instance, oleamide is an endogenous FA amide that resists biohydrogenation but can be digested in the intestines and can change the FA profile of milk in lactating dairy cows. According to Jenkins (1998), its consumption caused a drop in DMI but increased C18:1 in milk by 37% compared to the response of feeding the same amount of oleic canola acid and feeding a control diet, meaning less BH (T. C. Jenkins, 1999).

Perfield (2004) tested the effect of Amide-Protected and Lipid-Encapsulated Conjugated Linoleic Acid (CLA) supplements on milk fat synthesis. The amide-protected supplement contained CLA as an FFA, 27.2% of 18:1 *cis*-9, 24.7% of 18:2 *cis*-9, *trans*-11 and 21.1% of 18:2 *trans*-10, *cis*-12. As expected, milk fat yield was reduced on the first days of treatment and returned to normal when finished. These findings are similar to other studies where *trans*-10 *cis*-12 were abomasally infused or provided intravenously (Perfield et al., 2004).

Microbe Resistant Shell

Encapsulation with aldehyde treatment

This method is about protecting fat by using the formaldehyde treatment of a lipid-protein matrix, which allows the modification of the fatty acid composition of ruminant milk and feeding the cows a higher amount of fat without disturbing ruminal fermentation since formaldehyde protects the fats from BH. However, due to its acidic environment, the

formaldehyde-protein complex is hydrolyzed in the abomasum, and the fat can be available and absorbed in the small intestine. For this method, fat emulsification using proteins is the first step before adding aldehyde. The most common proteins used are casein and gelatin, but proteins from plants, fish, meat, and oilseed are also used. They create a homogeneous distribution of the fat into the protein that will serve as a coating that will be applied using spray drying.

This technique can change the fatty acid profile of milk, plasma, and muscle after 24 to 48 hours of diet administration, increasing PUFA levels from 2-5% to 20-30%, according to Scott et al. (1971). In an in vitro study, Gulati tested the efficacy of fats embedded in a matrix of aldehyde-treated protein, comparing them to calcium salts, extruded pelleted fat, prilled fat, and untreated fat. Untreated fat ruminal degradation was 95%, which was the highest, followed by extruded fat at 70%, prilled fat at 65%, calcium salts at 45%, and capsuled fat at 15% (Gulati et al., 1997). Protein capsules were used to protect fatty acids and other nutrients. To test their efficacy, treated and untreated capsules were filled with 10 g of trans-10 cis-12 CLA and tested orally on lactating dairy cows for 11 days. The performance of capsules was also compared with Calcium Salts. Treated capsules and Calcium salts caused MFD, but only the calcium salts could transfer a significant amount of the CLA into milk fat (Myers et al., 2005).

The University of California researchers had filed patents for protecting lipids and other nutrients by using a heated protein matrix to create a rumen-protected gel. The study consisted of testing those capsules vs. unprotected ingredients. As a result, protected gel treatment increased linoleic acid in milk by 3.25% on average (Jenkins et al., 2007). In

China, Guo et al. (2017) used capsules containing niacin, K2SO4, vitamin C, and gamma-aminobutyric acid to test if it could reduce the negative effect of heat stress on lactating dairy cows. They found that rumen-protected capsules alleviate the adverse effects of heat stress.

One disadvantage of the use of formaldehyde is that its use is not allowed in the European Union to feed milking cows because it can be noxious, and its use is limited to feed cows that produce milk for the swine or the cosmetic industry (Food & Authority, 2014). In the United States, it can be used under certain conditions. More recent methods have been proven to work without any fear of toxicity because the remains of formaldehyde are removed with an extra washing step (Jenkins, 2009). Thus, formaldehyde-treated protein capsules decreased the BH of CLA in *in vitro* tests (Myers et al., 2005). However, its use is limited due to its high cost and possible residues in the final animal products.

Scott et al. (1971) successfully protected FA from BH in vitro and *in situ*; therefore, many authors started to report formaldehyde-treated fats (Mattos et al., 2000; Jenkins et al., 2007; Gulliver et al., 2012). It was Gulati et al. (2005) who condensed some studies to conclude that the transfer efficiency (from diet to end products) of C 18 PUFA using this method was higher than for C 22 PUFA (19-44% vs. 10-14%). It would be proved later that using formaldehyde-treated linseed, BH would be significantly lower than if linseed oil was not protected (24.3 vs 43,5% respectively), and the levels of C 18:3 n-3 in plasma and milk were higher in the protected treatment compared to non-protection (Sterk et al., 2010).

Non-enzymatic browning.

Richardson proposed a different method of protection, it consists of cross-linking proteins with reducing sugars. This protection can be accomplished in three steps.

First, an aqueous emulsion of oil in a solution of protein and reducing sugars (such as lactose) is made. Then, those emulsions are freeze-dried to obtain a dry powder, and last, the powder is browned in an oven to produce rumen-protected granules (Richardson, 1992). Other authors have suggested encapsulating PUFA using an outer coating of Maillard reaction products for non-ruminal applications (Subramanian et al., 2012). According to Richardson (1992), this process is less effective and can also be expensive for using sugars and heating steps. Finally, the high temperatures needed for an effective Maillard-type reaction can affect the FA and generate toxic subproducts (Rosenberg et al., 2010).

Lipid composite gels

Gelatinization of the dispersion of lipid droplets in an aqueous protein phase can be used to protect lipids from ruminal BH. The making up of these lipid-protein gels takes two steps. First is to form an emulsion by mixing lipids in a matrix of dissolved or suspended proteins; then, the emulsion is heated up to 125°C to produce the gel. The proteins used in this method usually come from whey, blood serum, peanut, cereal, fish, or soy, which is an advantage compared to the methods that involve formaldehyde. However, it has a limitation related to the low shell life in storage for the deterioration caused by the

large amounts of water contained (van Vuuren et al., 2010). However, some authors consider them stable (Weinstein et al., 2016).

Carroll et al. (2006) published the first data obtained from gel protection. They were able to increase the content of C18:2n-6 in milk after one week of administration. That represented an efficiency of 30.1% compared to unprotected oil. They also reported no increase in *trans* C18:1 intermediate. Later, it was found by Weinstein et al. (2016) that feeding whey protein isolate gels complexes of soybean/linseed oil increased PUFA and decreased *trans-FA* levels in milk and plasma. During this study, the highest (43%) net transfer of C18:3 n-3 to milk occurred using whey protein isolate. Whey protein concentrate was lower (9%) than evening calcium salts (9%). Its effect was tested later in more extended periods (10 weeks), increasing the transfer efficiency of C 18:3 n-3 up to 19% in dairy cows (van Vuuren et al., 2010).

Encapsulation with lipids

Besides methods using proteins, other forms of protection, like microcapsules of lipids, are created under one of the two concepts. Active compounds are either embedded in a lipid matrix or formulated in small spheres later coated with lipids (Papas et al., 1997). Lipids' use as protection has always been related to the hydrophobic coating, where particles comprise a core with the protected substance and a hydrophobic coat of hydrogenated fats that completely encapsulates the core (Klose, 2012). The results of using this method are comprehensive and depend on the inner core (Lorenzon, 2015) despite sharing the same composition principle. One of the most significant advantages of this

method is the low cost compared to the methods using polymers, and its most crucial disadvantage is the low amount of material that can be protected per load and its limited post-ruminal release. One commercial product that uses this coating type is Lutrell® from BASF (Ludwigshafen, Germany), which contains *trans*-10, *cis*-12 C18:2.

Other encapsulation techniques

Jenkins (2007) declared that a method of protection could involve polymeric microspheres, using a polymer such as poly-lactide or another aliphatic polyester that is resistant to the rumen but is biodegraded in the acidic pH in the abomasum or hydrolyzed in the intestines. According to them, 50% of the capsules with this protection would survive the ruminal conditions for at least 24 hours. Jay et al. (2006) reported effective ruminal protection when using this method but very poor degradation in the abomasum. Another polymeric coat has been used by Akashe et al. (2014), but this one includes two layers. The first inner coat is of zein or caseinate. The second layer is a delayed-release material like gum Arabic, gelatin, ethylcellulose or hydroxypropyl methylcellulose. Nano-encapsulation is another proposed method of ruminal protection, and it has been tested, but its preparation is not completely clear (Heo et al., 2016).

Efficiency of Rumen-Protected Fats.

The efficiency of protected fat relies on the principle of bypassing the rumen but allowing intestinal release for further absorption of PUFA's into the lymphatic system and transfer to the peripheral tissues (Gadeyne et al., 2017). However, understanding the flow

and efficiency of rumen-protected fats is complicated because of all the methods and data available. Each one of those methods has positive and negative sides. *In vivo*, methods can be done in fistulated animals to determine how consuming rumen-protected fat sources affects the unsaturated fatty acid concentration in milk and duodenal flow. On the other hand, *in vitro* cultures are faster and cheaper, but they often need to be re-scaled in particle size. They also produce unrealistic data on the rate of fatty acid metabolism (Jenkins et al., 2007).

To find a way to increase n-3 PUFA in milk, protected fats have been compared to unprotected fats, finding that both methods could be used to achieve the goal. However, protected fat supplementation is more efficient since it prevents BH from impacting intake or milk yield (Kitessa et al., 2004). On the other hand, even though feeding rumen-protected fat can increase the energy supply for the cow, it can decrease glucose availability, which is a precondition for high milk production (Lohrenz et al., 2010).

Factors Affecting Rumen Degradation

Physical factors

Even though rumen DM digestibility depends mainly on factors like the animal itself and the ingredients of the diet (Maulfair et al., 2011), the particle size of the feed has been shown to impact ruminal fermentation because it has a significant effect on the rate of passage (Murphy et al., 1984). Kennedy (1985) observed the difference in rumination between chopped forage and forage in pelleted form. They found that due to the particle size, the first diet required 28,000 to 36,000 chews/day; meanwhile, the pellets needed from

3,000 to 10,000 chews/day. The size of the feed also determines how long it will stay in the rumen, and at the same time, the time spent in the rumen determines how well-digested the feed will be before leaving the rumen. For a long time, it was believed that particles larger than 1.18 mm would stay longer in the rumen, increasing rumen digestion. Later studies (Maulfair et al., 2011) showed that 3.5 mm would be critical for particles to remain longer in the rumen of high-producing dairy cows. However, the Penn State particle separator, the most known for particle size classification, still works under the premise that bigger particles than 19 mm will form the rumen mat and have the highest effect on stimulating rumination. Particles between 7.5 and 19 mm have a moderate rate of digestion and flow. Finally, particles between 1.18 and 7 mm are digested or pass more rapidly (Kononoff et al., 2003).

The effect of particle size on gas production has also been tested. Gerson (1988) found that particles between 1 and 2 mm had a lower fermentation rate than particles from 0.1 to 0.4 mm; bigger particle sizes were up to 600% more fermented than smaller ones. Lipolysis and hydrogenation occurred faster in bigger particle sizes due to the higher population density attached per area.

Rumen pH

Fatty acids inhibiting milk fat synthesis in the mammary gland are produced due to an incomplete rumen BH when high amounts of unsaturated FA are fed. One of the causes of the presence of those FAs is the alteration of microbial processes in the rumen that can occur because of pH variations. It was found that fiber digestion was reduced when

lowering the pH from 6.4 to 5.6. Total VFA, branched-chain VFA, concentrates of acetate, and butyrate were decreased as well (Fuentes et al., 2011).

Bacteria are sensitive to low pH, and changes can reduce their growth. Fibrolytic bacteria are susceptible, and when it is affected by pH, it reduces the access of other bacteria and enzymes to protein, increasing the dietary N flow and reducing the CP degradation. Low pH can also change the DNA concentration of bacteria involved in lipolysis and the processes of BH (Fuentes et al., 2011).

Temperature

The "inner body temperature" of the cattle under thermoneutral conditions is between 38 and 38.5°C. An animal's main metabolic heat-producing organs are the heart, liver, kidneys, and brain. However, in ruminants, fermentation is responsible for 3 to 8% of the total heat generated in the gut under a regular diet. Dale (1954) found that the temperature in the rumen is usually 2°C higher than the rectal temperature. However, in fasting conditions, the difference is only 0.7°C. Under hot conditions, sweating and panting start as a mechanism to maintain homeostasis, and DMI decreases, which reduces fermentation and helps to keep the temperature as low as possible (Beatty et al., 2008).

The temperature of the rumen varies depending on where the temperature is taken from; the top and the middle of the rumen stay around 40°C, but the bottom stays about 38.5°C (Beatty et al., 2008). Dale (1954) also found that consumption of low-temperature water reduced the temperature of the rumen. After water intake at 17°C, the rumen

temperature decreased to around 24°C, followed by an increase of temperature in the next 5 mins after consumption and only reached the normal temperature after about two hours.

Evidence showed (Gengler et al., 1970) that the concentration of volatile ruminal FA's is affected by ambient temperatures. When the ambient temperature is high, acetic acid production decreases the most compared to other VFAs. Thus, it affects the rumen ratio of acetic-propionic. The same research tested the effect of high temperature in the rumen, comparing control to other treatments that placed two intra-ruminal heating coils, one at 43.4°C and the other at 51.0°C. They found that DMI would decrease compared to a control group, but no changes in the concentrations of VFA were observed. Thus, differences in VFA cannot be explained only by the higher temperatures in the rumen. Later, Bhatta et al. (2005) would test the effect of temperature on fermentation by using an *in vitro* system. They tested a normal temperature (39°C) and a higher one (41°C), finding that temperature would negatively affect digestibility only numerically because there was no statistical difference. It was concluded that more than the temperature, pH is responsible for the most significant changes in fermentation.

As heat stress has repercussions on animal health and yield, cold stress has been reported to negatively affect the immune system of calves, fat deposition, meat quality in beef cattle, and milk yield in dairy cows. When the temperature is too low, the energy requirements increase to maintain body temperature. Cold stress increases rumination activity, increasing the passage rate and reducing digestion (Kang et al., 2019).

In ruminants, water intake decreases when water is cold and near 0°C. However, it did not show a significant effect on the percentage of dry matter digestibility, crude protein digestibility, and crude fiber digestibility (Brod et al., 1982)

It is unclear if long periods of high Temperature (>41°C) in the rumen would affect microbial populations and rumen fermentation. However, it is known that rumen protozoa do not survive those high temperatures (Beatty et al., 2008)

Summary

Differing from non-ruminants, on ruminants, dietary fat has a minor effect on milk fat composition. Due to its caloric density value, fat is commonly used as an energy supplement in ruminants because otherwise, its consumption would be limited (Hess et al., 2008). Producers have to be careful with the total dietary fat provided; it should not exceed 6 to 7% of the diet DM (Jenkins 1993); in general, fat intake can be increased up to 0.75 kg/d, but it could cause some repercussions, such as a decreased DMI, however, milk yield could not result affected, nor protein, or fat content (Beaulieu et al., 1995).

Fat supplementation could directly affect milk alteration levels of urea, protein, calcium, FA profile, and flavor. (Palmquist et al., 1993). These changes are made mainly on milk's long-chain FA (>18 carbons) and could be wider depending on the breed. In 1981, Karijord found a genetic variation within breeds towards the fatty acid composition of milk fat. Grummer in 1990 demonstrated differences in milk fat composition from Holsteins and Jerseys during week 7 to week 14 under the same diet (Grummer, 1991). The reaction of bacteria to unsaturated fatty acids is apparently the same among the breeds.

Contrary to Saturated fatty acids, unsaturated fatty acids are toxic for certain groups of bacteria, mainly the ones involved in fiber digestion. Microbes use BH as a detoxification reaction. *Trans* 10-C18:1 is an intermediate product of BH in the rumen that affects milk fat yield by reducing the amounts of mRNA for lipogenic genes in tissue. It inhibits the action of the enzymes in charge of the synthesis of short and medium-chain fatty acids by the mammary gland.

Research has focused on protecting fat from the rumen environment to avoid this reaction. Generally, "Protected fats" refer to all the fats designed to resist ruminal biohydrogenation and flow to the duodenum (Jenkins et al., 2007). Omega 3 can enhance cardiovascular and anti-inflammatory properties, among other health benefits.

By protecting these fatty acids, those properties could be transferred to milk. Thus, this study aimed to determine the effect of feeding protected omega 3 FA on milk fat composition.

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Figure 1.1. The change in percentage (%) from control of milk fatty acid composition for Holstein cows receiving three different amounts of added dietary fat: .25, .50, and .75 kg/d. Adapted from (Beaulieu et al., 1995)

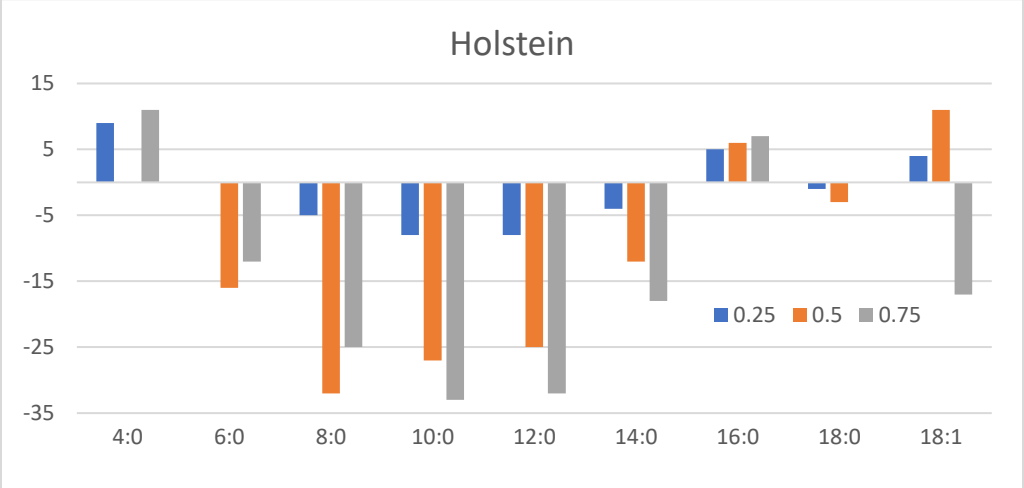

Figure 1.2. The change in percentage (%) from control of milk fatty acid composition for Jersey cows receiving three different amounts of added dietary fat: .25, .50, and .75 kg/d. Adapted from (Beaulieu et al., 1995)

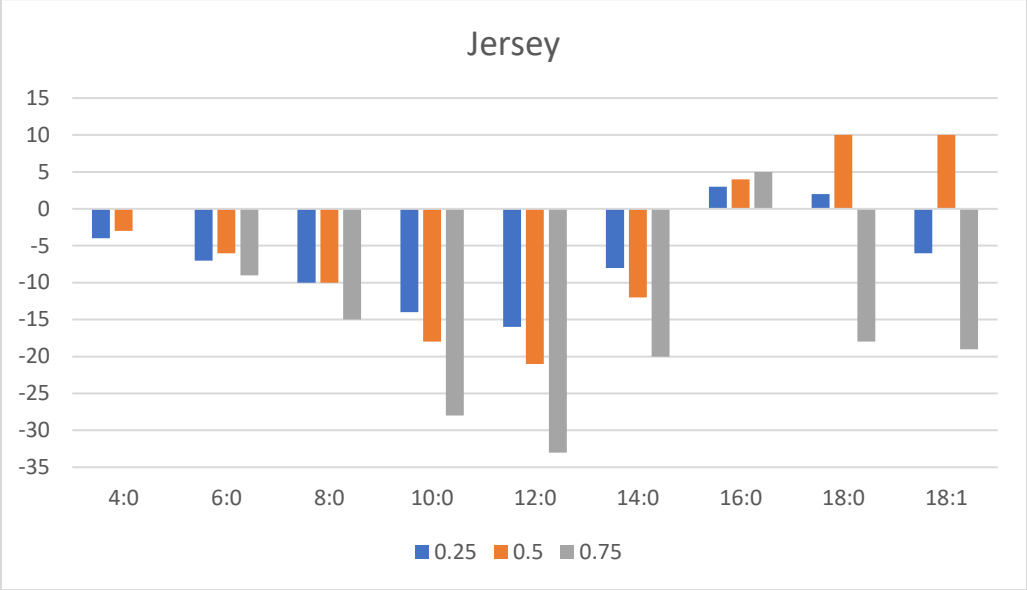

Figure 1.3. The biohydrogenation pathways of (A) α-linolenic, (B) linoleic, and (C) oleic acids. Adapted from Jenkins et al. (2008).

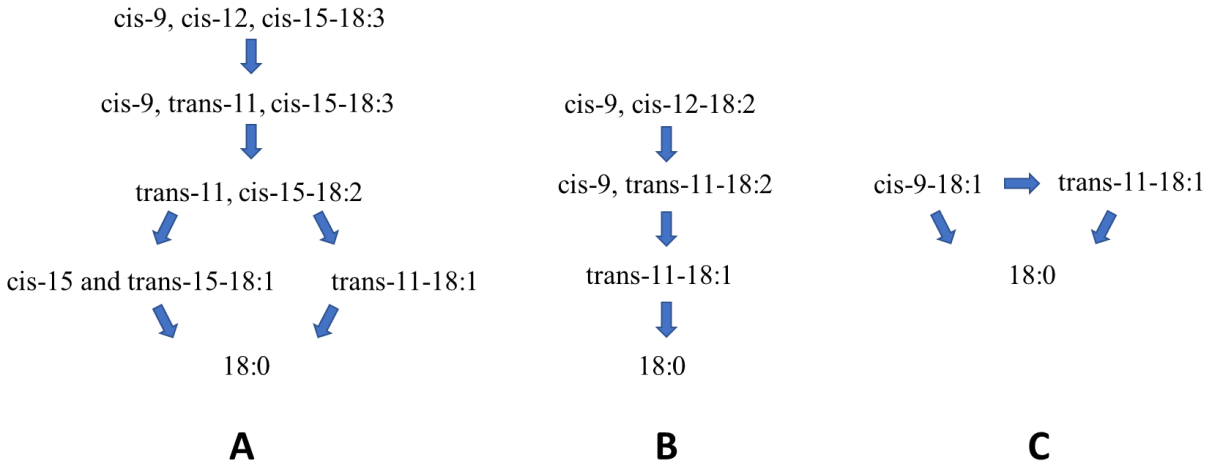

Figure 1.4. Pathway of ALA as precursor of other omega 3 FA, from feedstuff to EPA. Adapted from Gulliver et al. (2012).

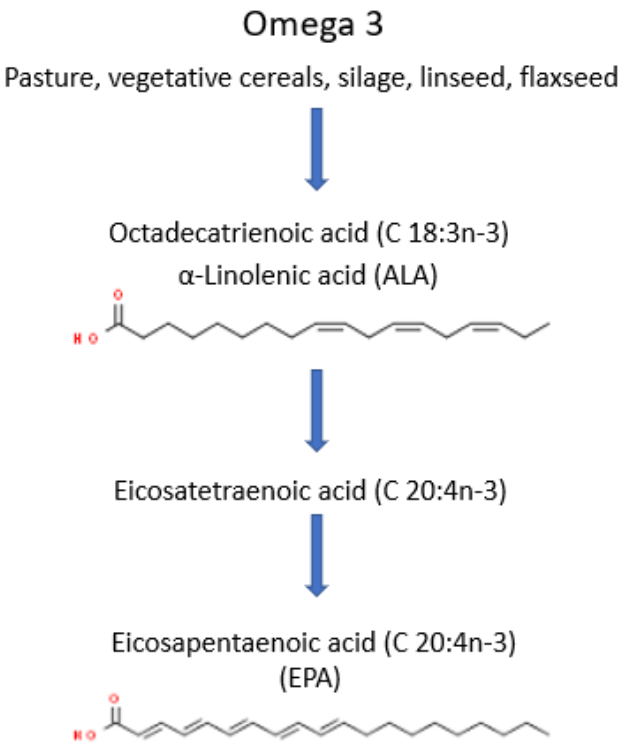

Figure 1.5. Mean values of relative differences in the digestibility of individual fatty acids. Adapted from Lock et al. (2006).

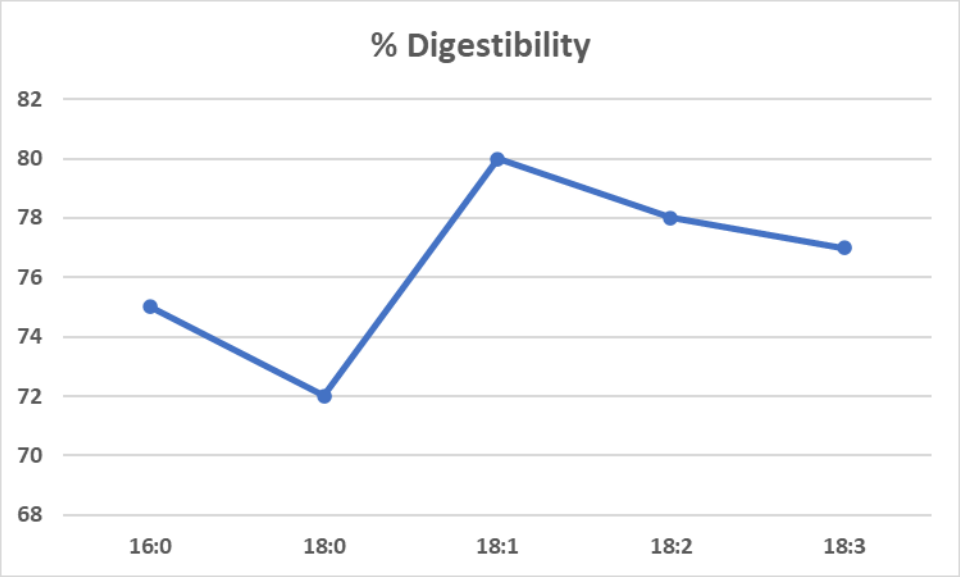

Figure 1.6. Example of Calcium salt with alpha-linolenic acid, that is, Ca (octadeca-9,12,15-trienoic acid) (Gadeyne et al., 2017).

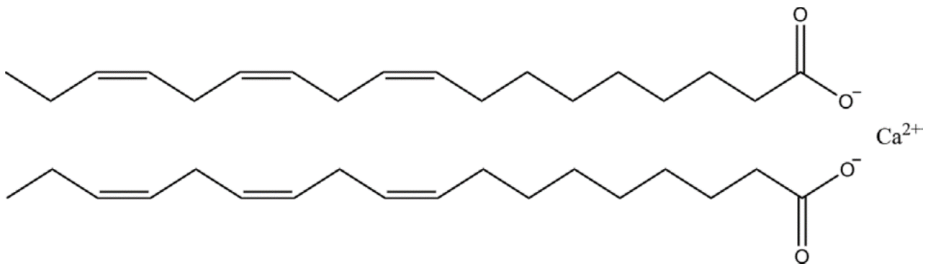

Figure 1.7. Example of a fatty acyl amide with alpha-linolenic acid, that is N-butyl-octadeca- 9,12,15-trienamide (Gadeyne et al., 2017).

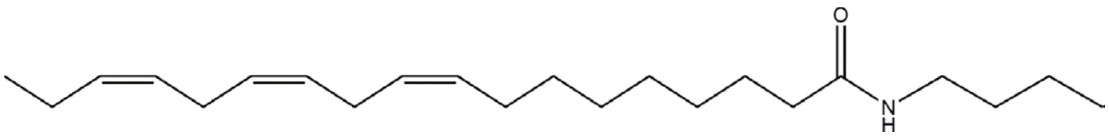

Table 1.1. Milk fat and fatty acid composition of different breeds compared to Holstein. Adapted from Soyeurt et al. (2006).

	Breed			
Milk fat and fatty acids (g/dL of milk)	**DPB[1]**	**RED[2]**	**MON[3]**	**JER[4]**
FAT	-0.48	-0.12	0.56	1.46*
Saturated Fatty Acids	-0.56	-0.01	0.73*	1.60*
Monounsaturated Fatty Acids	-0.47	-0.40	0.08	0.95*
12:0	-0.11	0.32	0.97*	1.37*
14:0	-0.30	0.13	1.12*	1.40*
16:0	-0.69*	-0.15	0.50	1.16*
16:1	-0.54	-0.52*	-0.29	0.43
18:0	-0.49	-0.08	0.68*	1.45*
18:1	-0.21	-0.31	0.11	0.88*
18:2	-0.09	-0.23	0.19	1.25*

[1]Dual Purpose Belgian Blue.
[2]Red and White.
[3]Montbeliarde.
[4]Jersey.
*Statistically difference with a *P-value* ≤ 0.05.

CHAPTER TWO

EVALUATING THE RUMEN DEGRADATION OF NOVEL PROTECTED GELATIN CAPSULES CONTAINING FISH OIL FED TO LACTATING DAIRY COWS

INTRODUCTION

Increased intake of polyunsaturated fatty acids (PUFA) has been associated with multiple beneficial effects in human health, including reduction in susceptibility to cardiovascular (Palmquist, 2009) and autoimmune diseases (Hahn et al., 2022). However, due to the rumen biohydrogenation process, most of the PUFA fed to ruminants is hydrogenated to more saturated forms (Jenkins et al, 2008). Thus, only a small percentage of the PUFA consumed by cattle is absorbed in the intestine and further incorporated into milk and meat. Supplementation of dairy cow diets with fish oil, which contains a large amount of unsaturated, bioactive n-3 fatty acids (FA) including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) has increased the concentration of *cis*-9, *trans*-11 CLA and long chain n-3 PUFA in milk (Kairenius et al., 2015). However, DHA supplementation can also increase the levels of *trans*-18:1 in the rumen with a concomitant decrease in milk fat yield caused by fish oil FA increasing rumen biohydrogenation intermediates that inhibit milk fat synthesis (Kairenius et al., 2015, Loor et al., 2005, Moallem, 2018). Several methods of protecting PUFA from rumen metabolism have been investigated extensively with the objective of improving milk production, reproduction, digestion, metabolism, and immunity (Gadeyne et al., 2017). For example, calcium salts are often reported to be one of the main commercially practical rumen protection formulations that allow the protection of polyunsaturated FA against rumen

biohydrogenation even though its efficacy is debated. Formaldehyde is one of the oldest cross-linking agents used to covalently bond proteins (Fraenkel-Conrat et al., 1948). Heating feeds that contain reactive amines and free sugars have been widely used to reduce the solubility of CP and increase the RUP fraction of various feedstuffs (Broderick et al., 1980). A similar approach has been evaluated to reduce rumen fermentation of glucose (McCarthy et al., 2020). However, these methods also have several disadvantages including protection by dissociation in the rumen at pH below 6.3 (calcium salts), toxicity and cost (formaldehyde and heating). In addition, there is a large variation in transfer efficiencies of dietary PUFA to milk, both between and within protection techniques. We propose a novel alternative rumen protection method where the target nutrient is contained within a protein (gelatin) capsule, treated with flavoring agents and heat, that enables resistance to dissolution in ruminal contents, but will also allow the gelatin to break down post-ruminally. One of the advantages of this novel method includes the possibility of loading the capsules with multiple nutrients (e.g., essential amino acids, essential FA, and pharmaceuticals). In addition, there is no chemical modification of the added nutrients. Recently, Jenkins (Jenkins et al., 2018) reported that treating capsules containing fish oil with the aforementioned method reduced their disintegration rate in the ruminal fluid and minimized changes in the fatty acid profile of the fish oil content under in vitro conditions.

Additionally, extensive capsule disintegration occurred that allowed rapid release of FA when treated capsules were exposed to pancreatic proteases. However, in situ methods cannot replicate the different conditions to which the capsules will be exposed from the time they are mixed in the diet and during their passage through the ruminant

gastrointestinal tract. We hypothesized that feeding fish oil in gelatin capsules treated for rumen protection will yield higher concentrations of EPA and DHA in milk than feeding fish oil in untreated gelatin capsules, without impairing animal performance, when fed to lactating dairy cows. For this proof-of-concept study, two trials were conducted in different locations with the objective of assessing the effects of feeding untreated and treated gelatin capsules containing fish oil on lactation performance, rumen FA content and milk enrichment of FA.

MATERIALS AND METHODS

Animals and Treatments

Trial 1. This study was conducted at the PhdR&D Research Institute, located in Fort Atkinson, WI, USA. Four multiparous ruminally-fistulated Holstein cows (means ± SD; 140.2 ± 56.6 DIM) were randomly assigned to one of four dietary treatments sequences in a 4 x 4 Latin square design with 21-d periods. Treatments consisted of 1) Control with no capsules (CO), 2) Control plus 200 untreated capsules per cow/day and mixed with the TMR (UF); 3) Control plus 200 per cow/day of treated capsules placed directly into the rumen (TR), 4) Control plus 200 treated capsules per cow/day and mixed with the TMR (TF). Capsules were mixed by hand in the TMR during the morning and afternoon feeding. The number of capsules not consumed by the cows was not recorded in this trial. Each capsule contained 140 mg of EPA, 65 mg of DHA, 16.5 mg of Oleic acid (18:1n9), 10.5 mg of Palmitoleic acid (16:1n7), and 9.5 mg of Docosapentaenoic acid (DPA). Thus, each

cow was supplied with 28 g of EPA and 13 g of DHA per day. The rumen protected capsules used in this study were treated with alcoholic solutions of flavoring agents followed by drying.

Relative proportion of dietary ingredients and chemical composition of the diet are reported in Table 2.1. The first 7 days of each period were a washout week with no treatment added to the rations, followed by 7 days of adaptation and sample collection performed during the final week. The length of the adaptation and sampling periods in this trial is not unusual and is found in several studies (Baumgard et al., 2002), (Carroll et al., 2006), (Beaulieu et al., 1995), (Lock et al., 2007). Cows were housed in tie-stall barns bedded on rubber mats with chopped pine shavings as bedding and had free access to water throughout the experiment. Diets were offered as total mixed rations (TMR) twice daily allowing for 5% refusals. Ingredient mix was adjusted based on forage DM analysis conducted 3 times per week. Care and handling of animals used for the study was conducted as outlined in the guidelines of the IACUC protocol number 2018_15.

Trial 2. The study was conducted at the LaMaster Dairy Center, Clemson University, located in Clemson, SC, USA. Three multiparous Holstein (means ± SD; 620 ± 25.0 kg of BW; 155 ± 18 DIM) and three multiparous Jersey (427 ± 7.3 kg of BW; 120 ± 22 DIM) ruminally fistulated lactating cows were randomly assigned to one of three dietary treatments sequences in a replicated 3 x 3 Latin squares design with 21-d periods. Treatments consisted of 1) Control with no capsules fed to the cows (CON); 2) Control plus 180 untreated capsules per cow/day (UC); 3) Control plus 180 treated capsules per cow/day (TC). Capsules were mixed by hand in the TMR during the morning and afternoon

feeding. Each capsule contained 91.6 mg of EPA and 75 mg of DHA. The number of capsules not consumed by the cows was recorded daily. On average, cows consumed 170 capsules when fed the UC and TC treatments. Thus, each cow was supplied with 15.58 g of EPA and 12.75 g of DHA per day.

Relative proportion of dietary ingredients and chemical composition of the diet are reported in Table 2.1. Cows were housed in tie-stall barns bedded on rubber mats with chopped wheat straw as bedding and had free access to water throughout the experiment. Diets were offered as total mixed rations (TMR) twice daily allowing for 5 to 10% refusals. Ingredient mix was adjusted based on weekly forage DM analysis. Care and handling of animals used for the study was conducted as outlined in the guidelines of the Clemson University Committee on Animal Use (AUP2019-017).

Sampling and Analyses of Feed and Milk

Trial 1. Samples of the TMR and feed refusals were collected twice per week, immediately dried at 55°C (forced-air oven) for 48 h, ground to pass a 1-mm Wiley mill screen (Arthur H. Thomas, Philadelphia, PA, USA) and composited by period. Samples were analyzed by Cumberland Valley Analytical Services (Waynesboro, PA, USS) for N (AOAC International, 2006), NDF using α-amylase and sodium sulfite (Van Soest etal., 1991), ADF (AOAC International, 2000), ether extract (method 954.02; AOAC International, 2000)(International, 2000), and starch (Hall, 2009). Individual cow DMI was computed weekly based on daily records of TMR offered and refused and the 105°C DM contents of the TMR and refusals.

Milk production was recorded on each cow at each of the 3 daily milkings (4 am, 12 p, 8 pm) throughout the study and summarized on a weekly basis. Milk samples were collected on 9 consecutive milking on d 19, 20, and 21 of each period. One sub-sample was collected in a bottle with preservers for analysis and determination of fat and protein by infrared analysis (AgSource Laboratories, Menomonie, WI, US). A second sub-sample was collected in a bottle with no preserver, composited by cow by day and stored at -20°C for analysis of FA. Total FA concentration and FA profiles of milk samples were analyzed at Cumberland Valley Analytical Services (Waynesboro, PA, USA). Milk lipids were extracted with hexane:isopropanol, transmethylated in the presence of sodium methoxide and FA were quantified by gas chromatography (Clarus 590 GC, PerkinElmer, Waltham, Massachusetts, USA) with a flame ionization detector and a 100mx 0.25mmx 0.2µl Supelco SP-2560 column. Average daily concentration and yield of milk components were computed using three milkings production as weighting factor. Yield of fat corrected milk (FCM) was calculated as 0.4*milk prod (kg/d) + 15 *(milk prod/100) *milk prod (kg/d) according to NRC (2001).

Trial 2. Daily samples of approximately 0.5 kg of the TMR and feed refusals were collected and stored at -20°C. Samples of silages and premixes were collected weekly. Feeds, TMR and refusals samples were dried at 55°C (forced-air oven) for 48 h and ground to pass a 1-mm Wiley mill screen (Arthur H. Thomas, Philadelphia, PA, USA). Samples were analyzed at Cumberland Valley Analytical Services (Waynesboro, PA, USA) for N, NDF, ADF, ether extract and starch concentration as described for Trial 1. Individual cow

DMI was computed weekly based on daily records of TMR offered and refused and the 55°C DM contents of the TMR and refusals.

Milk production was recorded on each cow at each of the 2 daily milkings (0600 and 1800 h) throughout the study and summarized on a weekly basis. Milk samples were collected from six consecutives milking on days 18, 19 and 20 of each period. One sub-sample was collected in a bottle with preservers and analyzed for fat and true protein by infrared analysis (Lancaster Dairy Herd Association, Manheim, PA, USA) with a Foss FT6000 (Foss North America Inc., Eden Prairie, MN). A second subsample from each milking was collected in a bottle without preserver, composited by cow and period for FA analyses. Milk samples were analyzed at Cumberland Valley Analytical Services (Waynesboro, PA, USA) for total FA concentration and FA profiles as described for Trial 1. Average daily concentration and yield of milk components were computed using morning and evening milk production as weighting factor. Yield of fat corrected milk (FCM) was calculated as 0.4*milk prod (kg/d) + 15 *(milk prod/100) *milk prod (kg/d) according to NRC (2001).

Sampling and Analyses of Rumen Fluid

Trial 1. Rumen fluid samples were collected on d-21 of each period 2 h after feeding and stored at -20°C until analysis for FA at the Department of Animal Science of Penn State University, PA, USA. Frozen rumen samples were lyophilized (Virtis 3.5 L XL, The Virtis Co.) and then ground to approximately <1 mm using a spinning-blade coffee grinder (model 80335R, Hamilton Beach, Glen Allen, VA, USA). Fatty acids were directly

methylated with sodium methoxide followed by methanolic HCL and extracted in hexane (Jenkins, 2010). Total FA concentration and FA profiles of rumen samples were determined by GC with FID according to Baldin et al., 2018 with 13:0 FFA, 17:1 TG, and 19:0 FAME as internal standards. For statistical analyses, FA concentrations from each sampling point were averaged per cow and per period.

Trial 2. Rumen fluid samples were collected from different locations in the rumen at 0 (pre-feeding), 1, 2, and 4 h after feeding on day 21 of each period and stored at -20°C until analysis for FA. Frozen rumen samples were lyophilized and analyzed for total FA concentration and FA profiles as described for Trial 1. For statistical analyses, FA concentrations from each sampling point were averaged per cow and per period.

Cow Body Weight and Blood Sampling and Analyses

Trial 2. Individual cow BW was recorded once at the beginning of the trial and at the end of each period. Blood samples were collected from each cow every 12h on day 19 of each period via jugular venipuncture using Serum Z/9-mL Luer Monovette collection tubes (Sarstedt Inc., Newton, NC, USA). Blood samples were allowed to clot at room temperature for about 1 h and then stored for 24 h at 4°C. All blood samples were centrifuged at 2,000 × g at 4°C for 20 min. Serum was then collected into separate tubes and stored at -20°C until analysis. Duplicate 1 mL serum aliquots from all steers were lyophilized (HarvestRight, North Salt Lake, UT, USA) and then transmethylated according to Tipton et al., 2020. An internal standard, methyl tricosanoic (C23:0) was incorporated into each sample during methylation. Each sample of fatty acid methyl esters was analyzed

using a Shimadzu GC-2014 gas chromatograph equipped with a Shimadzu AOC-20s automatic sampler. Separations were completed using a 60 m high resolution gas chromatography column (Agilent Technologies, Inc., Santa Clara, CA, USA). Samples were run at a split ratio of 10:1. Fatty acids were identified by comparing the retention times of known standards.

Statistical Analyses

Data were analyzed with the mixed procedure of SAS (SAS version 9.4, SAS Institute Inc., Cary, NC). For Trial 1, the statistical model included the effect of the period (1 to 4), the effect of the cow (1 to 4), the treatment effect (1 to 4), and the residual error. All terms were considered fixed, except for cow and the residual error. Treatment effects were evaluated by the following pre-planned orthogonal contrasts: (1) effect of control vs. fish oil capsules (UF + TR + TF), (2) effect of untreated fish oil fed directly into capsule (UF) vs. treated fish oil capsules (TR + TF), and (3) effect of treated fish oil capsule fed directly into the rumen (TR) vs. treated fish oil capsule mixed with the TMR (TF).

For Trial 2, the model included the effect of square (i.e., breed, 1 to 2), the effect of the period (1 to 3), the effect of cow within square effect (1 to 4), the treatment effect (1 to 3) the interaction between square and treatment, and the residual error. All terms were considered fixed, except for cow (within square) and the residual error. Treatment effects were evaluated by the following pre-planned orthogonal contrasts: (1) effect of Control vs. fish oil capsules (UC + TC), and (2) effect of untreated fish oil capsule (UC) vs. treated

fish oil capsule (TC). For both trials, significance was declared for $P \leq 0.05$ and tendency for $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

One cow was removed from the study during the last experimental week of Trial 1 because of health issues unrelated to the dietary treatments. No samples were taken from her for this period. On Trial 2, one cow, assigned to UC treatment, was removed from the study during the last week of period 1 because of health issues (mastitis) and was replaced with a non-fistulated cow. According to our statistician, the model does not change because of the removed cows. This situation produced some missing data, which is not an issue since our model (Latin Square Design) allows us to have this type of inconvenience without causing an effect on the responses we are measuring. Finally, for the measurements reported below for Trial 2 (Tables 2.3, 2.5, and 2.8), the interaction between capsules treatments and cow breed did not reach significance and therefore results will be presented as main effects of treatment and main effects of cow breed.

Feed Intake and Performance

In trial 1, compared with CO, we observed a tendency for a reduction in DMI (29.4 vs. 28.1 kg/d) and FPCM (45.6 vs. 41.5 kg/d) and a significant decrease in fat concentration (3.40 vs. 2.87%) and yield (1.80 1.43 kg/d) when feeding the fish oil capsules (Table 2.2). When compared with the untreated capsules, feeding either TF or TR tended to increase FCM and fat concentration. Furthermore, fat yield and FCM/DMI were significantly lower

for UC compared with both treated capsule feeding methods. Performance variables reported in Table 2.2 were unaffected by feeding the treated capsules directly into the rumen or by mixing them with the TMR.

In trial 2, DMI intake was not affected by the dietary treatments (Table 2.3). However, when compared with CON, milk yield was higher when cows were fed fish oil capsules, regardless of capsule treatment. Consequently, a tendency for an increase in milk/DMI was observed when cows were fed fish oil capsules. Performance variables reported in Table 2.3 were unaffected by feeding either untreated or treated capsules.

The negative effect of feeding fish oil on intake is largely dependent on the amount of oil fed and its characteristics, one of them is the smell produced when is directly mixed in the diet (Donovan et al., 2000). However, the reduction in intake observed in Trial 1 was unexpected because the amounts of fish oil fed in the trial were below the ones reported in the literature that might have an impact on feed intake (Moallem, 2018). Furthermore, feeding fish oil capsules will presumably reduce or eliminate the smell as a detrimental factor for DMI. The increase in milk production observed in Trial 2 is also puzzling, as most studies have shown that fish oil supplementation has no effect on milk yield. In Trial 1, adding fish oil capsules to the diet or directly into the rumen decreased milk fat content and yield, which is a typical response that has been frequently reported in the literature (Tipton et al., 2020, Donovan et al., 2000, Keady et al, 2000b), but it was also observed that treated capsules tended to mitigate to some extent the negative effect of feeding fish oil on milk fat concentration and yield. In Trial 2, the lack of treatment effect on milk percentage and yield suggests that either treated capsules broke in the rumen but the

amount of fish oil released was not enough to cause MFD, or the treated capsules did not dissolve in the ruminal fluid, but they were also not digested in the intestine, as indicated by the profile of fatty acids in blood. We recovered several pieces of treated capsules during ruminal sampling indicating that although the capsule shell did not dissolve, it did not remain intact after ingestion and during rumen passage. Thus, we hypothesis that abrasion in the rumen broke the capsule shells but the released oil did not penalize milk fat synthesis. Donovan (2000), found no difference in milk fat percentage in cows fed 290 g/d of fish oil compared to cows under no supplementation. Moreover, several authors (Donovan et al., 2000, Rego et al., 2005, Toral et al., 2010) observed a negligible fish oil effect when fed at higher doses than the one used in this study.

As expected, Holstein cows have significantly higher DMI, milk yield, and FCM yield than Jersey cows (Table 2.3). These results were consistent with previous studies comparing both cow breeds when fed a TMR diet (Pirondini et al., 2015, White et al., 2001). The cow breed did not influence feed efficiency, but the concentration of milk fat and protein in Jersey cows was numerically higher than in Holstein cows. The increase in fat and protein content in Jersey cow milk has been frequently reported in the literature (Pirondini et al., 2015, White et al., 2001). Due to the large difference in milk production, yield of fat and protein tended to be higher for Holstein cows.

Rumen Fluid Fatty Acid Composition

Table 2.4 presents the effects of the dietary treatments on the total fatty acid concentration in the rumen fluid for Trial 1. Compared with CO, the addition of fish oil

capsules to the diet decreased the concentration of C18:0 while numerically increasing *cis*-9 C18:1 by 38 and 10%, respectively. Additionally, feeding untreated or treated fish oil capsules consistently increased *trans*-6/8 C18:1, *trans*-9 C18:1, *trans*-10 C18:1, *trans*-11 C18:1, and *trans*-12 C18:1 concentration, compared with CO. Furthermore, most of the increase in total *trans* C18:1 fatty acid was due *trans*-10 C18:1 which along with *trans*-11 C18:1 is the last inter-mediate product in two different paths of rumen biohydrogenation of linoleic acid. The increased levels of those fatty acids can be explained by the amount of 18:1n9 present in the capsules. Adding fish oil capsule to the diet has a small impact on *cis*-9, *trans*-11 C18:2 CLA concentration. Finally, regardless of the method of administration of the capsules or the protection treatment, feeding fish oil capsules increased DHA content in rumen fluid. However, the amount of DHA detected for untreated capsules was numerically higher compared with treated capsules independent of the delivery method. This finding led us to think that treated capsules were physically stronger and did not release their content in the rumen at the same rate as untreated capsules. The capsule protection treatment or feeding method (rumen vs. feed) had no effect on the concentration of any of the reported rumen FA.

Table 2.5 presents the effects of the dietary treatments on the total fatty acid concentration in the rumen fluid for Trial 2. As we observed on trial 1, compared with CON, the addition of fish oil capsules to the diet decreased the concentration of C18:0 while tended to increase *cis*-9 C18:1 by 20 and 14%, respectively. Similarly, the concentration of total *trans*-C18:1 FA was increased by 39% when fish oil capsules were added to the diet. Adding fish oil capsules to the diet has no impact on *cis*-9, *trans*-11 C18:2

CLA and *trans*-10, *cis*-12 C18:2 CLA concentration. Compared with CON, DHA and total n-3 concentrations in ruminal fluid were higher when feeding untreated or treated capsules. The capsule protection treatment had no effect on the concentration of any of the reported rumen FA, except for a lower DHA concentration compared with. Rumen fluid collected from Jersey cows had a lower concentration of C14:0, C15:0 and C17:0, and a higher concentration of DHA.

Studies supplementing dairy diets with fish oil have also reported a reduction in C18:0 concentration in ruminal digesta in vivo (AbuGhazaleh et al., 2002) and under in vitro conditions (AbuGhazaleh et al., 2004) as a result of an incomplete biohydrogenation of unsaturated fatty acid. Furthermore, the increase in ruminal *cis*-9 C18:1 and *trans*-18:1 isomer when fish oil is supplemented to the diet, as we observed in both trials, is well documented in the literature (Bainbridge et al., 2016, AbuGhazaleh et al., 2002). It has been proposed that this increase in *cis*-9 C18:1 and *trans*-C18:1 FA with added DHA and EPA may be caused by inhibiting the reductase activity of ruminal microorganisms (AbuGhazaleh et al., 2004). Consequently, the similar changes in fatty acid biohydrogenation that we consistently observed when feeding either the untreated or treated fish oil capsules provides further evidence that the capsule treatments were unsuccessful to prevent the release of fish oil in the rumen. However, the lower concentration of DHA in the rumen fluid of cows fed the TC treatment, compared with UC, suggests that the capsule treatment was partially successful to prevent the release of fish oil in the rumen.

Plasma Fatty Acid Composition

Table 2.6 presents the effects of dietary treatments on plasma FA for Trial 2. Surprisingly, the fatty acid composition of plasma was not greatly affected by dietary treatments. Compared with CON, feeding fish oil capsules numerically increased the concentration of DHA and reduced *cis*-9 C18:1 and C20:4n-6. Moallem (2013), reported a drastic increase in plasma DHA from cows fed encapsulated fish oil, which indicates successful transfer of this fatty acid from the diet into the blood. However, this higher level of transfer can be explained by the time that project lasted. In their study cows were fed 4.3 g/d of DHA from day 256 of pregnancy until parturition and 13.5 g/d from there to day 100 postpartum. Longer times of supplementation mean higher amounts of consumption (Zachut et al., 2010).

Milk Fatty Acid Composition

Table 2.7 presents the effects of the dietary treatments on the total concentration of milk FA for Trial 1. Compared with CO, most of the individual short-chain FA decreased or tended to decrease when adding untreated or treated fish oil to the diet, except for C4:0, which remained unchanged by the treatments. However, compared with CO, milk from cows supplemented with fish oil capsules had a 7.4 and 8.4% lower concentration of C16:0 and C18:0 (tendency), respectably. In addition, dietary treatments changed the concentration of several individual *trans* monoene isomers present in the milk. In particular, the addition of fish oil capsules to the diet increased *trans*-6/8 C18:1, *trans*-9 C18:1, *trans*-10 C18:1, *trans*-11 C18:1, and *trans*-12 C18:1 concentration in the milk.

Furthermore, fish oil supplementation increased *cis*-9, *cis*-12 C18:2n-6, *cis*-9, *trans*-11 C18:2 CLA, and *cis*-9, *cis*-12, *cis*-15 C18:3n-3 concentration by 10, 69 and 14%, respectably.

Regardless of the delivery method, the content of EPA and DPA in milk fat was 2.1 and 5.5 times higher, respectively, when feeding fish oil capsules. Overall, when compared with the untreated capsules, supplementing treated capsules had no effect on milk FA profile, except for a higher *cis*-9 C18:1 and lower *cis*-9, *trans*-11 C18:2 CLA. Furthermore, milk FA contents were largely unaffected by placing the treated fish oil capsules directly into the rumen or by mixing them with the TMR.

In trial 2, fish oil supplementation had a minor effect on the total concentration of most short and medium chain milk FA (Table 2.8). However, compared with CON, milk from cows supplemented with fish oil capsules had an 8.8 and 10.2% lower concentration of C15:0 and C16:0, respectably. Like in Trial 1, supplementing cows with fish capsules increased the concentration of *trans*-6/8 C18:1, *trans*-9 C18:1, *trans*-10 C18:1 (numerically), *trans*-11 C18:1, and *trans*-12 C18:1 in the milk. Likewise, adding fish oil capsules to the diet increased *cis*-9 C18:1, *cis*-9, *cis*-12 C18:2n-6, *cis*-9, *trans*-11 C18:2 CLA, and *cis*-9, *cis*-12, *cis*-15 C18:3n-3 concentration by 8, 13, 40 and 17%, respectably. Adding untreated or treated fish oil capsules to the diet increased total n-3 FA (C18:3n-3, C20:5n-3, C22:5n-3, and C22:6n-3) content by 38% despite not detecting the same increase in plasma. Most of this increase in n-3 FA was due to the increase in EPA and, to a lesser extent, to DHA.

The response to fish oil supplementation on milk fat composition is well documented in the literature and is consistent with the changes in milk fatty acid profile observed in Trials 1 and 2 (Kairenius et al., 2015, Loor et al., 2005, Bainbridge et al., 2016). For example, supplementation of EPA and DHA usually results in decreased milk fat levels of C18:0 and *cis*-9 C18:1 due to the incomplete biohydrogenation of polyunsaturated FA in the rumen (Kairenius et al., 2015, Loor et al., 2005). Furthermore, the higher milk fat concentration of *trans*-C18:1 that we observed in both trials is likely the result of DHA from fish oil enhancing *trans*-C18:1 production from other polyunsaturated FA and not the direct conversion of DHA into *trans*-C18:1 isomers (Klein et al., 2011). Finally, several studies have shown that fish oil supplementation consistently elevates milk C20:5n-3, C22:5n-3, and C22:6n-3 concentration. However, the lack of difference in milk EPA and DHA concentration and *trans*-C18:1 between the untreated and treated capsules, further demonstrates that the proposed protection treatment failed to prevent the breakdown of the capsules in the rumen.

We observed several significant differences in the composition of milk FA between the two breeds used in this study (Table 2.8). Data from each breed showed that Jerseys produced significantly higher concentrations of C6:0 than Holsteins. However, Holstein cows produced significantly higher concentrations of *cis*-9, *cis*-12 C18:2n-6 and tended to produce higher concentrations of C20:5n-3 EPA than Jerseys. Several of the fatty acid levels reported for the present study do not agree with previous work comparing Holstein and Jersey cows. For example, there was no difference between the two breeds in the total production of several short- and medium-chain FA (C:4 and C8:0 to C12:0) as reported by

White et al., 2001. Similarly, several studies (Pirondini et al., 2015, Zachut et al., 2010) have shown that compared with Jersey, Holstein cows have a higher milk content of mono-unsaturated FA and *cis*-9, *trans*-11 C18:2 CLA and in Holstein cows that is associated with the higher Δ9-desaturase activity in the mammary gland (Soyeurt et al., 2008). However, the interpretation of the results from this study should be done with caution due to the low number of evaluated cows from each breed, that might have precluded us from detecting significant differences.

Moisture Effect on the Shell Hardness of the Treated Capsules

During the rumen sampling procedures conducted in Trial 2, the number of rumen intact capsules were visually assessed. We observed several intact and numerous pieces of broken but not degraded treated capsules. However, no intact or pieces of the untreated capsules shells were recovered. Taken together, these observations suggest that the shells of the treated capsules might have suffered a transformation that made them weaker to friction, even though they would not degrade in the rumen. To test this theory, we conducted a trial consisting of submerging 12 treated capsules in a 39°C water bath for up to 120 minutes to measure their change in weight and hardness after different incubation lengths (0, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, and 120 min). One capsule was weighted and evaluated for harness at each time point. Capsule hardness was determined with a durometer (FstDgte, Shore A Durometer, Guilin Digital Electronic Co., Ltd., Guilin, Guangxi, China), which is the international standard to measure the hardness of rubber, plastic, and other non-metallic materials. Changes in capsule weight and hardness are illustrated in Figure 2.1. The smaller capsule weight was measured at time 0 (0.48 g), then

steadily increased during the incubation period, and after 120 minutes the capsule was 40% heavier than at the start of the incubation. On the contrary, the hardness of the capsule was highest at time 0, decline to 84% after only 10 min of incubation, and was negligible after 1 hour. These data suggest that when in contact with high levels of moisture, as it is expected when capsules are mixed with the TMR, saliva, and rumen fluid, the shell of the treated capsule was likely weakened, which probably led to the breakup of the capsule due to the abrasion at what it was exposed to in the mouth and the rumen.

CONCLUSIONS

In summary, results from these two studies indicated that gelatin capsules treated with alcoholic solutions of flavoring agents followed by drying containing fish oil for rumen protection did not result in higher concentrations of EPA and DHA in milk compared with untreated gelatin capsules, as we originally hypothesized. In addition, we observed that feeding untreated or treated fish oil capsules consistently increased the production of biohydrogenation intermediates, both in the rumen fluid and in milk fat, with a concomitant detrimental impact on milk fat concentration and yield in one of the trials. But in Trial 2, the lack of a significant impact on milk fat yield when feeding either the untreated or treated capsules prevents us from making any additional inference on the effectiveness of protection treatment. However, the mitigating effects on milk fat yield observed in Trial 1 when cows were fed treated capsules and the recovered pieces of broken treated shells in Trial 2 confirms the findings of Jenkins (2018) which indicated that the proposed protection method reduced the degradation rate of the capsule in the ruminal fluid. Further testing of the capsules revealed the susceptibility of the treated capsules to

abrasion because of a reduction of shell hardness when exposed to high levels of moisture. Thus, future studies are warranted to evaluate alternative methods such as coating to minimize the contact between the capsule shell and environmental moisture.

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Figure 2.1. Changes in weight and hardness after incubating treated capsules in water at 39°C.

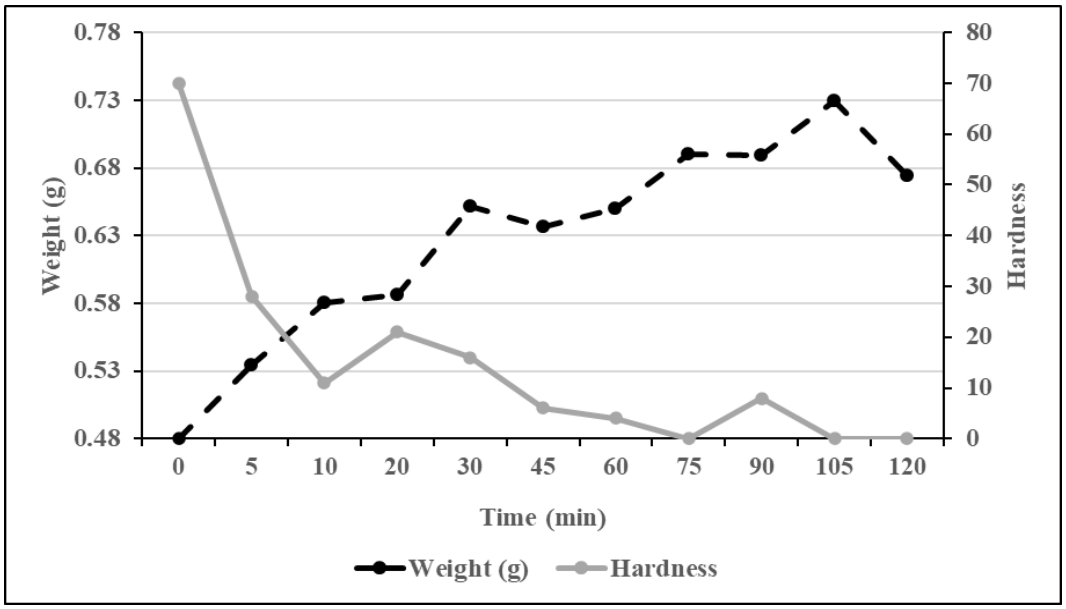

Table 2.1. Ingredient and chemical composition of control diets fed on Trial 1 and Trial 2.

	Control diet	
Item	**Trial 1 (CO)**	**Trial 2 (CON)**
Ingredient composition	(%DM)	
Corn Silage	33.3	40.0
Alfalfa Silage	18.6	--
Alfalfa Hay	--	10.0
Soy Hulls	5.3	--
Dry Corn	10.1	--
High Moisture Shell Corn	10.1	--
Whey	3.9	--
Premix Trial 1[1]	18.7	--
Premix Trial 2[2]	--	50.0
Chemical composition		
DM, as-is	41.2	50.3
CP % DM	15.5	15.9
NDF, % DM	29.7	33.0
ADF, % DM	20.3	21.2
NFC, % DM	45.0	40.5
Starch, % DM	25.5	27.6
Ether extract % DM	4.73	--
FA %DM	3.43	--
FFA, % DM	--	0.68

[1] Premix Trial 1 composition (DM basis): Canola meal 37.6%, Soyplus® 19.4%, Expeller meal® 19.4%, Kinetic® 6.6%, RP mix (Blood meal) 6%, sodium bicarbonate 4%, calcium carbonate 2.3%, white salt 1.2%, AB20 Bentonite® 1%, PHD High VTM® 0.9%, urea 0.6%, Mepron® 0.5%, magnesium oxide 0.4%, Diamond V XPC® 0.2%, Rumensin® 0.1%.

[2] Premix Trial 2 composition (DM basis): Ground corn 42%, soybean meal 14%, Soyplus® 13%, corn gluten feed 9%, soybean hulls 4.7%, whole cotton seed 5.1%, calcium Ca 2.9%, molasses 2.3%, sodium bicarbonate 2.2%, bentonite 0.8%, Palmit 80® 0.7%, magnesium oxide 0.6%, salt 0.6%, potassium carbonate 0.5%, potassium chloride 0.5%, urea 0.4%, trace mineral and vitamin mix 0.38%.

Table 2.2. Effect of dietary treatments on animal performance for Trial 1.

Item	Treatment[1]				SEM[2]	*P*-value[3]		
	CO	UF	TF	TR		1	2	3
DM intake, kg/d	29.4	28.2	27.6	28.4	1.1	0.07	NS	NS
Milk, kg/d	49.7	48.3	50.4	50.2	5.5	NS	NS	NS
4% FCM[4], kg/d	45.6	38.4	42.7	43.3	3.6	0.09	0.08	NS
Fat, %	3.40	2.60	2.96	3.04	0.13	0.02	0.07	NS
Protein, %	2.96	2.94	2.84	2.82	0.07	NS	NS	NS
Lactose, %	4.59	4.53	4.59	4.58	0.03	NS	0.17	NS
Fat, kg/d	1.8	1.3	1.5	1.5	0.13	0.01	0.02	NS
Protein, kg/d	1.5	1.4	1.4	1.4	0.17	NS	NS	NS
Milk yield /DMI[5]	1.68	1.68	1.83	1.77	0.08	NS	NS	NS
FCM/DMI5	1.53	1.33	1.55	1.52	0.06	NS	0.05	NS

[1]CON = Control with no capsule (CO); UF = Control plus 200 untreated capsules per cow/day and mixed with the TMR; TR = Control plus 200 per cow/day of treated capsules placed directly into the rumen; TF = Control plus 200 treated capsules per cow/day and mixed with the TMR.
[2]Standard error of the mean.
[3]$P \leq 0.2$ are shown for the contrasts: 1 = control vs. fish oil capsules (UF + TR + TF); 2 = untreated fish oil fed directly into the rumen (UF) vs. treated fish oil capsules (TR + TF); 3 = treated fish oil capsule fed directly into the rumen (TR) vs. treated fish oil capsule mixed with the TMR (TF).
[4]4% fat corrected milk.
[5]Efficiencies calculated as milk (kg/d) or FCM (kg/d) divided by DMI (kg/d)

Table 2.3. Effect of dietary treatments on animal performance for Trial 2.

Item	Treatment[1]			SEM[2]	P-value[3]		Breed[4]		SEM	*P*-value[3]
	CON	UC	TC		1	2	H	J		
DMI, kg/d	21.6	22.1	22.1	0.47	NS	NS	25.2	18.6	0.57	<0.01
Milk, kg/d	27.8	31.3	30.2	1.9	0.06	NS	35	24.5	2.4	0.04
4% FCM[5], kg/d	29.6	31.2	30.45	1.3	NS	NS	33.7	27.1	1.5	0.03
Fat, %	4.77	4.14	4.18	0.39	0.17	NS	3.99	4.73	0.42	NS
Protein, %	3.33	3.24	3.31	0.10	NS	NS	3.16	3.42	0.13	NS
Fat, kg/d	1.26	1.23	1.16	0.08	NS	NS	1.33	1.11	0.07	0.1
Protein, kg/d	0.88	1.00	0.97	0.06	0.13	NS	1.08	0.82	0.07	0.06
Milk yield/DMI[6]	1.28	1.41	1.36	0.08	0.06	NS	1.38	1.32	0.11	NS
FCM/DMI[6]	1.35	1.40	1.34	0.09	NS	NS	1.32	1.40	0.07	NS

[1]CO = Control with no capsules; UC = Control plus 180 untreated capsules per cow/day; TC = Control plus 180 treated capsules per cow/day.
[2]Standard error of the mean (highest when uneven samples).
[3] $P \leq 0.2$ are shown. The contrasts: 1 = Control vs. fish oil capsules (UC + TC); 2 = untreated fish oil capsules (UC) vs. treated fish oil capsule (TC).
[4]H = Holstein; J = Jersey.
[5]4% fat corrected milk
[6]Efficiencies calculated as milk (kg/d) or FPCM (kg/d) divided by DMI (kg/d).

Table 2.4. Rumen FA profile of lactating cows fed the dietary treatments in Trial 1.

	Treatment[1]				SEM[2]	*P*-value[3]		
FA, g/100 g of total FA	**CO**	**UF**	**TF**	**TR**		**1**	**2**	**3**
C14:0	1.10	1.11	1.07	1.15	0.04	NS	NS	NS
C15:0	0.61	0.60	0.61	0.67	0.02	NS	NS	NS
C16:0	27.8	26.8	26.8	25.4	0.8	NS	NS	NS
C17:0	0.40	0.38	0.39	0.39	0.01	NS	NS	NS
C18:0	31.7	20.9	23.3	24.5	2.5	0.03	NS	NS
trans-6/8 C18:1	0.54	0.96	0.95	1.05	0.09	0.01	NS	NS
trans-9 C18:1	0.28	0.75	0.70	0.77	0.12	0.02	NS	NS
trans-10 C18:1	1.16	3.96	3.84	3.64	0.95	0.07	NS	NS
trans-11 C18:1	2.57	4.44	4.04	4.30	0.4	0.02	NS	NS
trans-12 C18:1	0.79	1.11	1.19	1.25	0.06	<0.01	0.18	NS
Total *trans* C18:1	5.35	11.21	10.72	11.01	1.2	0.01	NS	NS
C18:1n-9	7.86	9.32	8.58	8.22	0.40	0.14	0.13	NS
C18:2n-6	9.43	10.92	9.74	9.33	1.2	NS	NS	NS
cis-9, *trans*-11 C18:2 CLA	0.068	0.132	0.128	0.123	0.02	0.05	NS	NS
trans-10, *cis*-12 C18:2 CLA	0.05	0.072	0.059	0.077	0.02	NS	NS	NS
cis-9, *cis*-12, *cis*-15 C18:3n-3	2.67	3.20	2.66	2.69	0.42	NS	NS	NS
C20:5n-3 EPA	N/D	0.54	0.2	0.14	0.2	0.18	0.14	NS
C22:5n-3	N/D	0.071	0.02	0.002	0.03	NS	NS	NS
C22:6n-3 DHA	N/D	0.44	0.23	0.26	0.10	0.05	0.19	NS
Total n-3	N/D	1.05	0.45	0.4	0.29	0.13	0.15	NS

[1]CON = Control with no capsule (CO); UF = Control plus 200 untreated capsules per cow/day and mixed with the TMR; TR = Control plus 200 per cow/day of treated capsules placed directly into the rumen; TF = Control plus 200 treated capsules per cow/day and mixed with the TMR.
[2]Standard error of the mean.
[3]$P \leq 0.2$ are shown for the contrasts: 1 = control vs. fish oil capsules (UF + TR + TF); 2 = untreated fish oil fed directly into the rumen (UF) vs. treated fish oil capsules (TR + TF); 3 = treated fish oil capsule fed directly into the rumen (TR) vs. treated fish oil capsule mixed with the TMR (TF).

Table 2.5. Rumen FA profile of lactating cows fed the dietary treatments in Trial 2.

	Treatment[1]			SEM[2]	P-value[3]		Breed[4]		SEM[2]	*P*-value[3]
FA, g/100 g of total FA	**CON**	**UC**	**TC**		**1**	**2**	**H**	**J**		
C14:0	0.87	0.84	0.84	0.03	NS	NS	0.9	0.8	0.02	0.04
C15:0	0.55	0.52	0.5	0.02	0.1	NS	0.55	0.5	0.01	0.07
C16:0	20.9	20.9	20.5	0.35	NS	NS	20.9	20.6	0.31	NS
C17:0	0.40	0.37	0.36	0.01	0.02	NS	0.39	0.36	0.01	0.04
C18:0	35.8	27.9	29.1	1.9	0.03	NS	32.3	29.5	2.0	NS
trans-6/8 C18:1	0.47	0.67	0.6	0.04	0.02	NS	0.58	0.59	0.04	NS
trans-9 C18:1	0.26	0.37	0.34	0.02	0.01	0.2	0.31	0.34	0.03	NS
trans-10 C18:1	0.85	1.41	1.0	0.22	NS	NS	1.27	0.9	0.21	NS
trans-11 C18:1	2.77	4.3	3.52	0.41	0.04	0.15	3.35	3.71	0.5	NS
trans-12 C18:1	0.76	1.04	0.96	0.04	0.01	0.2	0.9	0.94	0.04	NS
Total *Trans*	5.12	7.8	6.42	0.62	0.03	0.13	6.4	6.49	0.7	NS
cis-9 C18:1	7.5	8.7	8.4	0.41	0.1	NS	7.9	8.6	0.36	NS
cis-9, *cis*-12 C18:2n-6	14.2	15.8	15.2	0.77	NS	NS	14.4	15.8	0.67	NS
cis-9, *trans*-11 C18:2 CLA	0.09	0.16	0.14	0.04	NS	NS	0.13	0.14	0.04	NS
trans-10, *cis*-12 C18:2 CLA	0.12	0.11	0.08	0.02	0.17	0.15	0.12	0.09	0.02	NS
cis-9,*cis*-12,*cis*-15 C18:3n-3	1.86	1.96	1.99	0.01	NS	NS	1.91	1.96	0.08	NS
C20:0	0.59	0.99	1.06	0.04	<0.01	NS	0.83	0.93	0.04	NS
C20:5n-3 EPA	ND	0.44	0.23	0.07	0.2	NS	0.15	0.29	0.06	0.2
C22:6n-3 DHA	0.09	0.46	0.33	0.03	<0.01	0.02	0.20	0.38	0.04	0.04
Total n-3[5]	1.94	2.89	2.65	0.19	0.02	NS	2.28	2.71	0.19	0.19

[1]CO = Control with no capsules; UC = Control plus 180 untreated capsules per cow/day; TC = Control plus 180 treated capsules per cow/day.
[2]Standard error of the mean (highest when uneven samples).
[3]$P \leq 0.2$ are shown. The contrast 1 = Control vs. fish oil capsules (UC + TC); 2 = untreated fish oil capsules (UC) vs. treated fish oil capsule (TC).
[4]H = Holstein; J = Jersey.
[5]C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3.

Table 2.6. Plasma FA profile of lactating cows fed the dietary treatments in Trial 2

	Treatment[1]			SEM[2]	P-value[3]		Breed[4]		SEM[2]	*P*-value[3]
FA, g/100 g of total FA	**CON**	**UC**	**TC**		**1**	**2**	**H**	**J**		
C14:0	0.31	0.33	0.32	0.02	NS	NS	0.33	0.3	0.01	0.2
C16:0	6.2	6.5	6.1	0.24	NS	NS	6.5	6	0.25	NS
C18:0	10.2	9.6	9.3	0.34	0.12	NS	9.7	9.7	0.28	NS
cis-9 C18:1	3.5	3.1	3.1	0.17	0.04	NS	3	3.4	0.18	0.2
cis-9, *cis*-12 C18:2n-6	37.7	39.2	37	1.58	NS	0.15	40.4	35.6	1.9	0.15
cis-9, *cis*-12, *cis*-15 C18:3n-3	2.1	2	2	0.11	NS	NS	2.1	1.9	0.13	NS
C20:4n-6	1.75	1.24	1.13	0.20	<0.01	NS	1.58	1.17	0.27	NS
C22:6n-3 DHA	0.12	0.23	0.18	0.04	0.18	NS	0.17	0.18	0.03	NS

[1]CO = Control with no capsules; UC = Control plus 180 untreated capsules per cow/day; TC = Control plus 180 treated capsules per cow/day.
[2]Standard error of the mean (highest when uneven samples).
[3]$P \leq 0.2$ are shown. The contrast: 1 = Control vs. fish oil capsules (UC + TC); 2 = untreated fish oil capsules (UC) vs. treated fish oil capsule (TC).
[4]H = Holstein; J = Jersey.

Table 2.7. Milk FA profile of lactating cows fed the dietary treatments in Trial 1.

	Treatment[1]				SEM[2]	*P*-value[3]		
FA, g/100 g of total FA	**CO**	**UF**	**TF**	**TR**		**1**	**2**	**3**
C4:0	4.4	4.33	4.21	4.23	0.14	NS	NS	NS
C6:0	2.22	2.09	2.01	1.95	0.08	0.05	NS	NS
C8:0	1.22	1.15	1.11	1.04	0.05	0.07	NS	NS
C10:0	2.75	2.61	2.52	2.31	0.12	0.07	NS	NS
C12:0	3.14	3.02	2.97	2.74	0.12	0.13	NS	NS
C14:0	10.4	10.2	10.2	9.8	0.21	NS	NS	NS
C15:0	0.89	0.87	0.82	0.86	0.03	0.14	NS	NS
C16:0	33.7	31.4	31	31.2	0.35	<0.01	NS	NS
C17:0	0.42	0.43	0.43	0.43	0.01	NS	NS	NS
C18:0	7.64	6.84	7.35	6.8	0.27	0.06	NS	NS
trans-5 C18:1	0.0073	0.018	0.021	0.0069	0.004	0.12	NS	0.06
trans-6/8 C18:1	0.37	0.61	0.57	0.62	0.026	<0.01	NS	NS
trans-9 C18:1	0.20	0.45	0.42	0.48	0.03	<0.01	NS	NS
trans-10 C18:1	1.29	2.64	2.54	2.39	0.53	0.07	NS	NS
trans-11 C18:1	0.93	1.84	1.54	1.58	0.11	<0.01	NS	NS
trans-12 C18:1	0.53	0.82	0.8	0.81	0.019	<0.01	NS	NS
cis-9 C18:1	15.7	14.5	15.8	16.2	0.4	NS	<0.01	NS
cis-9, *cis*-12 C18:2n-6	2.45	2.65	2.71	2.72	0.05	<0.01	NS	NS
cis-9, *trans*-11 C18:2 CLA	0.54	0.98	0.83	0.93	0.031	<0.01	0.02	0.08
trans-10, *cis*-12 C18:2 CLA	0.0063	0.0013	0.0013	0.012	0.003	NS	NS	0.06
cis-9, *cis*-12, *cis*-15 C18:3n-3	0.53	0.59	0.6	0.62	0.02	<0.01	NS	NS
C20:5n-3 EPA	0.03	0.073	0.066	0.053	0.01	<0.02	NS	NS
C22:5n-3	0.04	0.071	0.062	0.061	0.05	NS	NS	NS
C22:6n-3 DHA	0.017	0.095	0.083	0.102	0.01	0.05	NS	NS
Total n-3	0.088	0.238	0.211	0.216	0.04	<0.01	NS	NS

[1]CON = Control with no capsule (CO); UF = Control plus 200 untreated capsules per cow/day and mixed with the TMR; TR = Control plus 200 per cow/day of treated capsules placed directly into the rumen; TF = Control plus 200 treated capsules per cow/day and mixed with the TMR.
[2]Standard error of the mean.
[3]$P \leq 0.2$ are shown for the contrasts: 1 = control vs. fish oil capsules (UF + TR + TF); 2 = untreated fish oil fed directly into the rumen (UF) vs. treated fish oil capsules (TR + TF); 3 = treated fish oil capsule fed directly into the rumen (TR) vs. treated fish oil capsule mixed with the TMR (TF).

Table 2.8. Milk FA profile of lactating cows fed the dietary treatments in Trial 2.

FA, g/100 g of total FA	Treatment[1]			SEM[2]	P-value[3]		Breed[4]		*P*-value[3]
	CON	UC	TC		1	2	H	J	
C4:0	3.79	4.07	3.84	0.13	0.07	0.04	3.79	4.01	NS
C6:0	1.96	2.07	1.93	0.04	0.12	<0.01	1.86	2.11	0.03
C8:0	1.21	1.28	1.18	0.05	NS	0.02	1.13	1.3	0.17
C10:0	2.86	2.95	2.73	0.2	NS	0.11	2.7	3	NS
C12:0	3.43	3.38	3.21	0.25	NS	NS	3.2	3.48	NS
C14:0	10.7	10.6	10.3	0.24	NS	NS	10.5	10.5	NS
cis-9 C14:1	0.86	0.78	0.82	0.07	NS	NS	0.81	0.82	NS
C15:0	0.85	0.76	0.79	0.03	<0.01	0.18	0.78	0.82	NS
C16:0	32.3	28.6	29.4	1.09	<0.01	NS	29.9	30.3	NS
cis-9 C16:1	1.44	1.38	1.45	0.05	NS	NS	1.43	1.41	NS
C17:0	0.60	0.56	0.59	0.02	0.11	0.18	0.58	0.59	NS
C18:0	10.6	10.4	10.6	0.43	NS	NS	10.4	10.7	NS
trans-6/8 C18:1	0.26	0.4	0.35	0.03	<0.01	NS	0.34	0.33	NS
trans-9 C18:1	0.21	0.32	0.29	0.02	<0.01	NS	0.27	0.27	NS
trans-10 C18:1	0.42	0.71	0.56	0.12	0.15	NS	0.65	0.47	NS
trans-11 C18:1	1.08	1.74	1.54	0.15	<0.01	0.12	1.36	1.56	NS
trans-12 C18:1	0.39	0.64	0.59	0.04	<0.01	NS	0.54	0.55	NS
cis-9 C18:1	15.9	16.9	17.4	0.54	0.02	NS	17.3	16.2	NS
cis-9, *cis*-12 C18:2n-6	2.56	2.94	2.85	0.13	0.06	NS	3.03	2.53	0.05
cis-9, *trans*-11 C18:2 CLA	0.46	0.67	0.62	0.06	0.01	NS	0.53	0.58	NS
cis-9,*cis*-12,*cis*-15 C18:3n-3	0.29	0.35	0.33	0.02	0.07	NS	0.35	0.29	0.12
C20:0	0.16	0.25	0.28	0.02	<0.01	0.16	0.21	0.25	0.15
cis-11 C20:1	0.04	0.11	0.11	0.01	<0.01	NS	0.08	0.09	NS
C20:5n-3 EPA	0.03	0.07	0.06	<0.01	<0.01	0.05	0.05	0.06	0.07
C22:5n-3	0.06	0.08	0.07	0.01	0.03	NS	0.07	0.07	NS
C22:6n-3 DHA	0.05	0.08	0.07	0.02	NS	NS	0.07	0.07	NS
Total n-3[5]	0.4	0.58	0.52	0.04	0.01	NS	0.51	0.49	NS

[1]CO = Control with no capsules; UC = Control plus 180 untreated capsules per cow/day; TC = Control plus 180 treated capsules per cow/day.
[2]Standard error of the mean (highest when uneven samples).
[3]$P \leq 0.2$ are shown. The contrast: 1 = Control vs. fish oil capsules (UC + TC); 2 = untreated fish oil capsules (UC) vs. treated fish oil capsule (TC).
[4]H = Holstein; J = Jersey.
[5]C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3.

CHAPTER THREE

EVALUATING CAPSULE CHANGES AND ASSESING A DIFFERENT TYPE OF PROTECTION

INTRODUCTION

After testing the change on the hardness, size, and weight of the capsules, as mentioned in chapter two, the results indicated that in contact with high levels of moisture, like in the TMR, saliva, and the rumen environment, the shell of the capsules were susceptible to breakage. In our studies, the capsules could have been staying longer in the rumen due to their increased size (as documented in chapter 1) which increased the chances for abrasion due to the sum of all the physical changes mentioned before. This condition could increase exponentially inside of the rumen due to the friction generated because of the normal contractions of this organ. For this reason, we conducted several tests on the capsules. First, we evaluated the effect of the exposure of the capsules to a total mixed ration (TMR). Secondly, we try to find a coverage that could lead us to prevent the capsule collapse before leaving the rumen. Conducting these tests had as objective to find a method of protection to avoid the effect of moisture on the treated capsules.

TMR Exposure.

The first approach to the physical changes of the capsules was measured by mixing 15 of them with the TMR used in the second trial described in chapter 2. After mixing, we recorded the changes in weight and hardness of the capsules. Measurements were done at 15 different timepoints, one per timepoint (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70 mins).

We found that the weight of the capsules mixed with the TMR did not change as much during the whole trial (Figure3.1). However, the change in hardness was more evident. It decreased by 75% (Figure 3.2). The small change in weight could be explained by the low amount of water the capsule could absorb from the TMR. As we will see in other trials, when the capsules are exposed to water, they allow the water in the shell, generating higher changes on their weight. However, that moisture contained in the TMR is enough to cause a high impact on the weight of the bare capsule.

Parafilm® M Cover.

The first approach we took to protect the shell and prevent capsule abrasion was using Parafilm® M sealing film (Bemis Company Inc. Neenah, WI, USA) which is an uncolored, flexible film made of thermoplastic non-toxic materials, commonly used in laboratories as a covering seal for flasks, test tubes, among others. According to its house laboratory description, this material is odorless and tasteless.

In the first run we recorded the weight of three bared capsules A,B, and C, (means ± SD; 0.4801 ± 0.009 grams). Their weight after being covered with Parafilm® M, (means ± SD; 0.5194 ± 0.014grams) and their weight after being exposed to 39°C water, at four different timepoints (10, 20, 30 mins, and, 8 hours). As shown in table 3.1, during the first 2 timepoints the capsules maintain their weight, meaning that no water was getting inside of the shell. At the 30-minute timepoint of exposure to 39°C water, the bare capsules increased their weight by 35%. However, at some point during between the 30 first minutes

and the 8 hour timepoint the protection was not efficient, and the weight of the capsules increased by ± 52%.

Due to the lack of information in terms of time from that first test, we conducted a second test. This time we used 16 timepoints (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 150, 180, and 210 mins). Two capsules were tested at each timepoint, one bare, and other covered with Parafilm® M. For this run we collected the initial weight of both the capsules, the ones that were going to be covered and the bared ones, the weight after covering them, the hardness, and the change in size at every timepoint (measured as the smallest area of a square that the capsule would need to fit when laying on a 1mm grid paper). As results (Table 3.2), we found that non-covered capsules increased their weight from 16% at the 10-minute timepoint up to 76% at 210 minutes (Figure3.3) while the covered capsules did not suffer any alteration on weight (Figure 3.4). The non covered capsules decreased in hardness as they increased in size while the covered ones remain similar on both characteristics.

Using Parafilm® M, was a successful method of protection for the capsules when tested on its ability to maintain water away from the shell avoiding alterations on weight and hardness.

Stearic and Palmitic Acid Test

The last test we ran was using different concentrations of stearic (C:18:0) and palmitic acid (C:16:0). We prepared three different mixes (A:50 - 50%, B: 75% – 25%, and 25% – 75%, Stearic (97% octadecanoic acid, ACROS ORGANICS®) and Palmitic

acid (98% hexadecanoic acid, ACROS ORGANICS®) respectively). These fatty acids are commercialized as solids, so the percentage of each acid was determined by weight in a total mix of 100 grams. The mixes were made in three different 250 ml Erlenmeyer flasks that were exposed to high temperatures (around 70°C) in a water bath until reaching melting point while stirring constantly. After having a liquid solution, the capsules assigned to each mix (n = 3) were immersed and rolled in to obtain an even layer of the mix on the surface of the capsule. Finally, the capsules were stored at room temperature overnight before testing their resistance to water at 39°C on 8 different timepoints, 15, 60, 120, 180, 210, 240, 270 mins and 72 hours. In every treatment, capsule number 1 was used to measure hardness. Capsules break as result of measuring their hardness, meaning that only the other two capsules were used to collect data of weight, size and final hardness. Both capsules in each treatment were weighted at every one of the determined timepoints and the hardness was measured only after the last one of the timepoints.

As shown in table 3.3, the weight of the capsules after being covered with the mixes increased substantially (around 45%). The cover with mix A was not effective and both capsules broke before reaching 120 minutes. The cover with mix B was successful for both capsules until 270 minutes but one of them broke before reaching the 72 hours of incubation. The capsule that completed the whole trial did not change in weight, and the other capsule maintained its weight before breaking before the last timepoint. As shown in table 3.3, the hardness of the one successful capsule after hours was similar to the one of capsule number 1 tested at the beginning of this single trial.

Both capsules covered with the last mix (mix C) were successful and their weight did not change though the whole trial. Their hardness were very consistent with the one measured from the capsule number one.

This form of protection showed us a successful and partially successful method of coverage (C and B respectively), however, this process might be hard to replicate on a bigger scale, which could limit its use. More research is needed in order to accomplish a form of protection that is both efficient and easy to apply.

Figure 3.1. Initial vs final weight (grams) of non-covered capsules after being mixed with the TMR during 15 different timepoints.

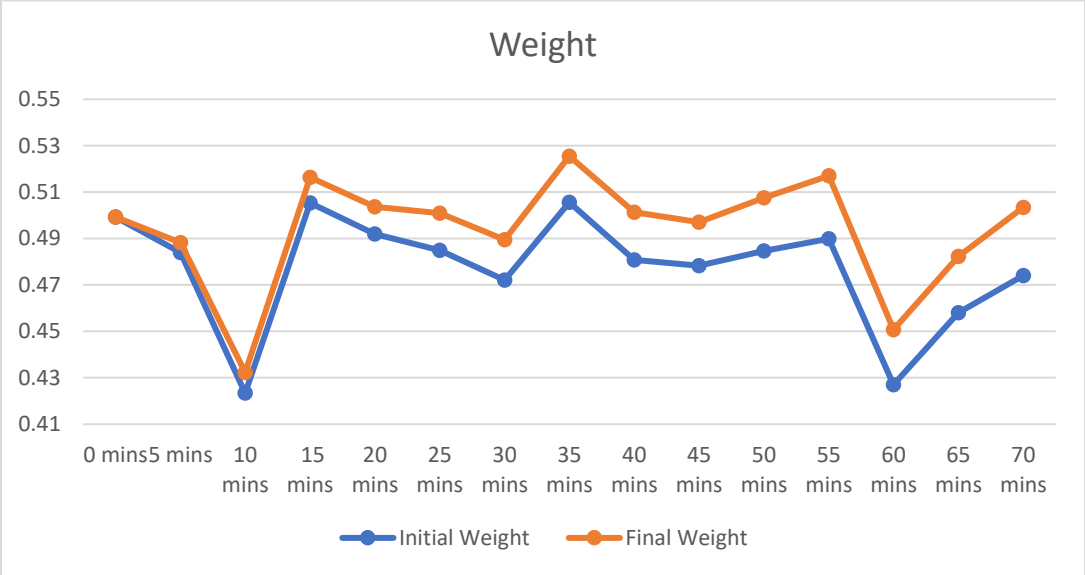

Figure 3.2. Change in hardness of non-covered capsules after being mixed with the TMR during 15 different timepoints.

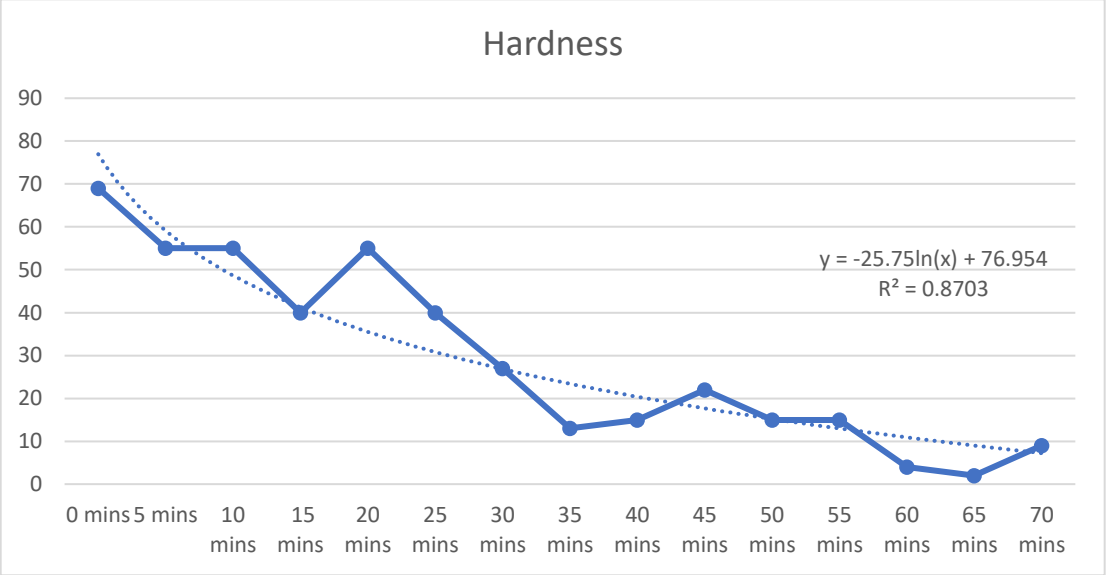

Figure 3.3. Initial vs final weight (grams) of non-covered capsules after 16 different timepoints of exposure to 39°C water.

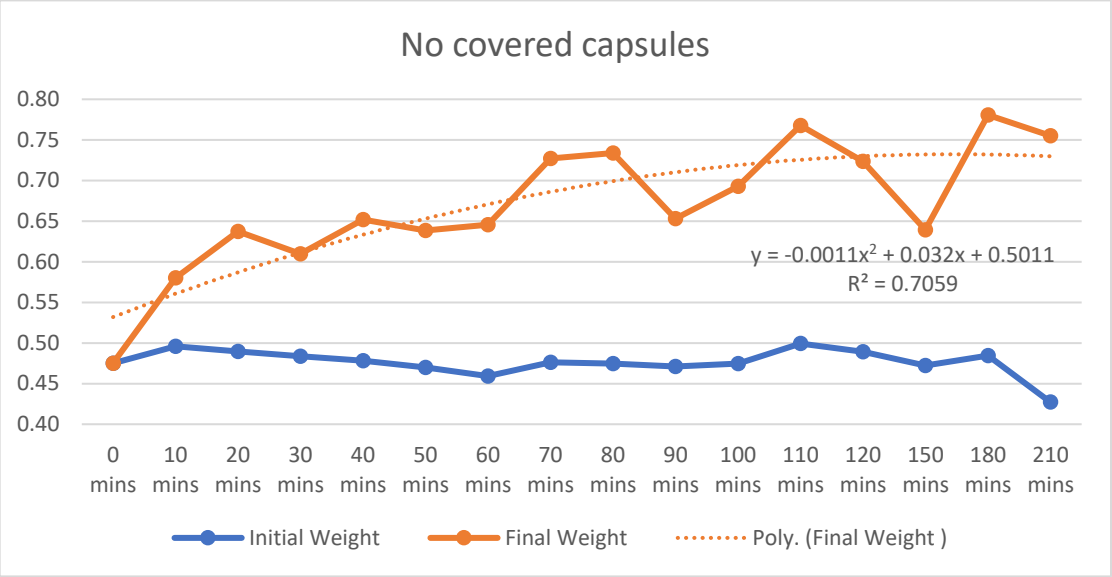

Figure 3.4. Initial vs final weight (grams) of capsules covered with Parafilm® M after 16 different timepoints of exposure to 39°C water.

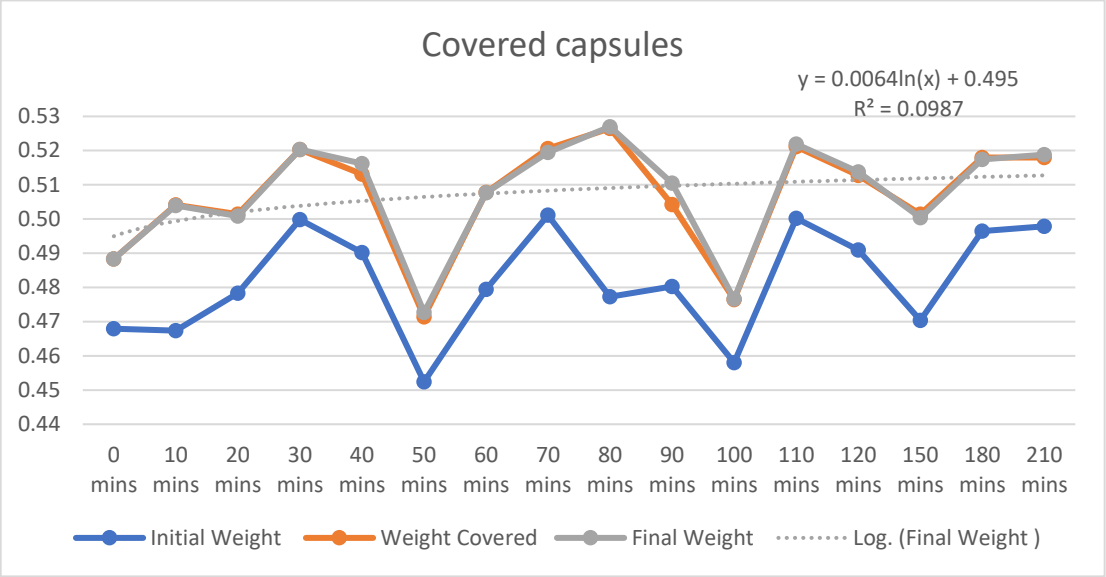

Table 3.1. Weight in grams of bare capsules at 4 different timepoints exposed to water at 39°C water.

Weight at	Capsule		
	A	B	C
0 mins	0.5188	0.5336	0.5057
10 mins	0.5269	0.5385	0.5056
20 mins	0.5322	0.5418	0.5041
30 mins	0.5366	0.5447	0.5065
8 hours	0.8518	0.8091	0.7238

Table 3.2. Changes in weight (grams), hardness, and size of the capsules, both covered and non-covered with Parafilm® M at 16 different timepoints after exposure to 39°C water.

Time-point (mins)	Non-covered Capsules				Covered Capsules				
	Initial Weight	Final Weight	Hardness	Size (mm)	Initial Weight	Weight + Parafilm® M	Final Weight	Hardness	Size (mm)
0	0.475	0.475	72.5	9 x 9	0.468	0.488	0.488	70.0	11 x 10
10	0.496	0.580	20.0	11 x 10	0.467	0.504	0.504	66.0	11 x 10
20	0.490	0.637	18.0	11 x 12	0.478	0.501	0.501	66.5	11 x 10
30	0.484	0.610	7.0	11.5 x 12	0.500	0.520	0.520	78.0	11 x 10
40	0.478	0.652	4.5	12.5 x 13.5	0.490	0.513	0.516	66.0	11 x 10
50	0.470	0.639	2.0	12.5 x 12.5	0.452	0.471	0.473	40.0	11 x 10
60	0.459	0.646	2.5	13 x 13.5	0.479	0.508	0.508	70.0	11 x 10
70	0.476	0.727	2.0	13 x 13.5	0.501	0.521	0.520	70.5	11 x 10
80	0.475	0.734	2.5	13 x 13	0.477	0.527	0.527	79.0	11 x 10
90	0.471	0.653	8.0	12 x 13	0.480	0.504	0.511	90.0	11 x 10
100	0.475	0.693	No read	11 x 14	0.458	0.476	0.477	78.0	11 x 10
110	0.499	0.768	No read	13 x 14	0.500	0.521	0.522	70.0	11 x 10
120	0.489	0.724	1.0	13 x 13	0.491	0.513	0.514	61.0	11 x 10
150	0.472	0.639	1.5	13 x 13	0.470	0.501	0.500	60.0	11 x 10
180	0.484	0.781	No read	13 x 15	0.497	0.518	0.517	48.0	11 x 10
210	0.427	0.755	2.0	11 x 14	0.498	0.518	0.519	79.0	11 x 10

Table 3.3. Change in weight and hardness of the capsules covered with different concentrations of Stearic and Palmitic acid (A:50 - 50%, B: 75% – 25%, and 25% – 75%, Stearic and Palmitic respectively).

Mix	A			B			C		
	1	2	3	1	2	3	1	2	3
Weight of the capsule	0.50	0.48	0.50	0.51	0.46	0.47	0.49	0.50	0.49
Weight capsule + Cover	0.79	0.74	0.89	1.22	1.05	0.84	0.81	0.69	0.82
15 min	NA	0.75	0.89	NA	1.05	0.84	NA	0.81	0.69
60 min	NA	-	0.93	NA	1.05	0.84	NA	0.81	0.69
120 min	NA	-	-	NA	1.51	0.84	NA	0.81	0.69
180 min	NA	-	-	NA	1.05	0.84	NA	0.81	0.70
210 min	NA	-	-	NA	1.05	0.84	NA	0.81	0.69
240 min	NA	-	-	NA	1.05	0.84	NA	0.81	0.69
270 min	NA	-	-	NA	1.05	0.84	NA	0.81	0.69
72 Hours	NA	-	-	NA	1.05	-	NA	0.81	0.69
Hardness	92	NA	NA	77.3	77.3	-	89	89	87