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Impact of DDGS feeding on the composition of milk and baby Swiss cheese

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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CHAPTER 1. INTRODUCTION

Cheese is the common name given for a group of fermented, coagulated milk products produced in a great variety of flavors, textures, and forms. Cheese can also be described as thick protein gel structure occluding fat and moisture along with other materials (Guinee and O' Brien, 2010). Cheeses are known to be biologically and biochemically very dynamic and highly unstable (Fox and McSweeny 2004). About 8000 years ago, preserving the milk constituents by fermentation was the main objective of cheese making. From then until now, it has evolved as a part of a high quality and highly nutritious diet.

Production and Consumption

In United States, a huge amount of cheese is produced every year. In 2013, the total cheese produced (excluding cottage cheese) was approximately 11.1 billion pounds, which was 2% higher than production in 2012 (USDA-NASS, 2014). That same year (2013), the total amount of Swiss cheese produced out of 66 plants was 294 million pounds, which is 8.1% less than that produced in 2012 (320 million pounds from 59 plants) (USDA-NASS, 2014).

Swiss Type Cheese Composition

Swiss cheese, originally manufactured in Switzerland, comes under the category of hard to semi-hard cheese, with Emmental being the most prominent Swiss-type cheese (Frohlich-Wyder and Bachmann, 2007). Commonly used starter bacteria for Swiss cheese production are *Streptococcus salivarius,* sbsp. *thermophilus, Lactobacillus helveticus*, and *Propionibacterium freudenreichii* sbsp. *shermanii* (Kosikowski and Mistry, 1997). Starter

cultures have multiple roles during cheese making, including acid production in a controlled rate, suppression of non-starters, aiding in removal of moisture, and controlling physical, chemical and microbial variations (Reinbold 1972). Swiss-type cheeses have typical round glossy eyes, which vary in size from 1 to 3 cm. The characteristic nutty flavor and eye formation is achieved by propionic acid production via fermentation by *P. shermanii* (Kosikowski and Mistry, 1997). Composition of milk also plays a major role in the textural characteristics of Swiss cheese (Lawrence et al., 1984).

Swiss cheese is considered one of the most challenging cheeses to make well because of its distinct starter combination and peculiar cooking, fermentation, and ripening conditions (USDA, 1978). The goal of any cheese maker is to produce a cheese with typical flavor and structure and appropriate count of uniformly distributed, round, glossy eyes of desired size. More than 90% of Swiss cheese production in U.S. is in the rindless block form. Such cheeses have soft body with uniformly distributed eyes and mild flavor (Cakir and Clark, 2009). Traditional Swiss cheese is made from raw milk. In the U.S, Swiss cheese milk, however is commonly heat-treated (thermized) to 67°C for 20sec to kill any unwanted flora, then cooled to 32°C before the cultures are added (Cakir and Clark, 2009). The U. S federal standards of identity for Swiss cheese states that the cheese milk used may or may not be pasteurized, and requires Swiss cheese to be aged at least 60 days (21 CFR 133.195, FDA, April 2014). Reinbold (1972) mentioned that superior cheese is produced from thermized milk rather than fully pasteurized milk.

According to USDA (2013), there are 66 plants currently making Swiss cheese around in U.S. Though every plant has their proprietary make procedures, the general methodology of Swiss cheese manufacture is shown in the flow chart (figure 1.1). In the

U.S., a slight modification of traditional Swiss cheese is more common, and the result is baby Swiss cheese. Baby Swiss is similar in appearance and is made using the same starter cultures. However, the baby Swiss cheese curds are cooked at lower temperatures and have shorter curing times; this leads to more mild flavor and smaller eyes than in traditional Swiss cheese (Kosikowski and Mistry, 1997; Cakir and Clark, 2009). Cakir and Clark (2009) described the cooking temperature for baby Swiss is around 39°C, while that for the traditional Swiss is around 54°C. The standard of identity for baby Swiss cheese requires a minimum 45% of milk fat by weight of solids and maximum 43% moisture content by weight (ATCP, 1985). The composition of other related cheeses with eyes are provided in Table 1.1.

After sufficient heat treatment (or pasteurization), the cheese milk temperature is modified to 32°C for culture addition. Swiss cheese involves fermentation by lactic acid producing (*S. thermophilus, L. helveticus)* and propionic acid-producing (*P. freudenreichii)* bacterial cultures (21 CFR 133.195, FDA, April 2014). The purpose of each starter in the fermentation process and its role towards final product is explained later in this Literature Review. After around 30 minutes after culture addition, chymosin (rennet or clotting enzyme) is added in order to obtain a curd mass. This semi-solid curd is cut into smaller curd particles (1/4 inches; 0.635 cm) by using appropriate curd knives, and stirred to increase firmness and to expel whey (Cakir and Clark, 2009, 21 CFR 133.195, FDA, April 2014).

Table 1.1 Typical composition of common cheese with eyes from various references (adapted and modified from E. Cakir and S. Clark, (2009) originally from Kosikowski and Mistry, 1997, Fox et al., 2000, Liggett at al., 2008, Lawrence et al., 1984, Great Lakes Dairy Symposium Proceedings, 2010)

Cheese types	Fat (%)	Total Protein (%)	Salt (%)	pH	Body and Texture
Asiago	31	31	3.6	5.3	Dry, firm, crumbly
Baby Swiss	31	24	1.1*	NR	Semi hard
Edam	24	26	2.0	5.7	Semisoft to hard
Emmentaler/Swiss	31	28	1.2	5.6	Semihard to hard
Fontina	26	24	1.2	5.6	Semisoft to hard
Gouda	29	27	2.0	5.8	Semisoft to hard
Gruyere	30	30	1.1	5.7	Semisoft to hard
Havarti	27	25	2.2	5.9	Soft to Semisoft

* = Salt % of 1 month old baby Swiss cheese

NR = not reported

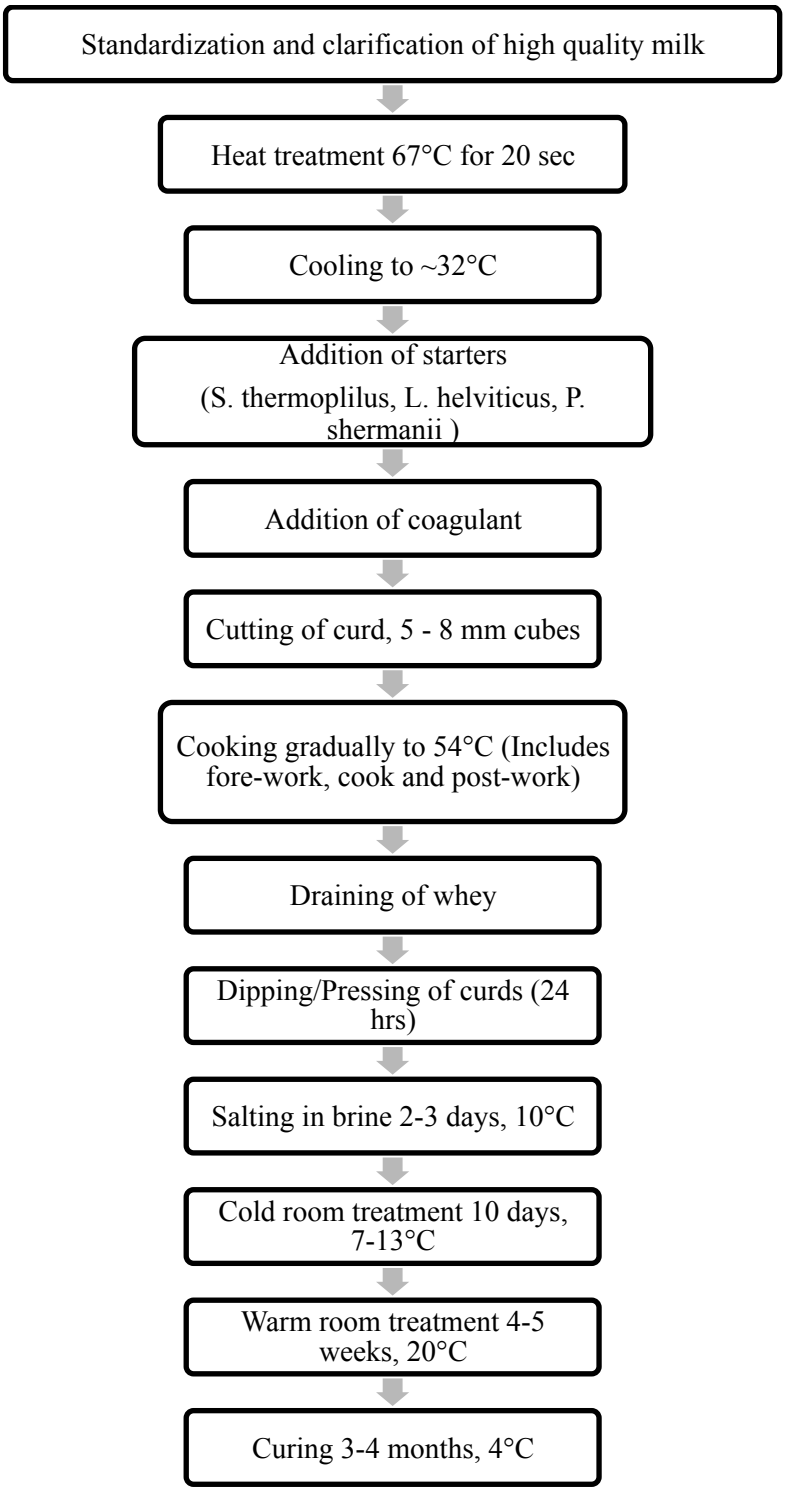

Figure 1.1: Flow chart showing Swiss cheese production (Adapted from Kosikowski and Mistry, 1997)

The temperature of curds is raised to ~54°C for Swiss, (~39°C for baby Swiss) through a step-by-step process, as follows. In the fore-work stage (1), the cheese curds are soft so must be gently mixed in the whey for about 40 min. Acid production by starter cultures helps in reducing the pH slightly to below 6.5. The cooking stage (2) involves increasing the temperature gradually to 39°C or 54°C within about 40 min. Higher temperature aids in making the curds firm, yet elastic, and therefore contributes towards the desired texture of the final product. In the post-work stage (3), depending upon the cheese curds' pH, acid concentration, moisture level and firmness, the curds are stirred for another 45 to 60 min. Acid production in the vat determines the basic characteristics of any cheese because the pH of the whey during draining is one of the very important factors that determine the pH of the final product. Lawrence et al. (1984), described that there are higher chances of good eye formation if the pH is slightly >6.2 during curd dipping.

The curds are collected and pressed inside a perforated mold overnight at about 20°C to obtain desired shape and firmness. The pressed cheese blocks are then removed and placed into a saturated (23%) NaCl and $CaCl_2$ brine solution for 2-3 days (Cakir and Clark, 2009). The NaCl promotes shelf life and improves flavor; the $CaCl_2$ (added about 0.2 to 0.3 g/100 g) prevents calcium leaching to the surface of the cheese (Johnson and Law, 2010). The pH of cheese after 24 hours should be 5.2 to 5.3 for desired eye formation (Lawrence et al., 1984); the salt content should be 0.7% to 1.1% w/w).

It is interesting to note that, among other major cheese varieties like Cheddar (1.5%, w/w) and Gouda (2.0%, w/w), Swiss cheese is the least (0.7% to 1.1%, w/w) salted while the highest salted variety is Domiati (6.0%, w/w) (Guinee and Fox, 2004). Cheeses are brined because the salt helps to form a natural rind on the surface of the block, which acts as a

protecting barrier against microflora (Hutkins. 2006). Propionibacteria are known to be sensitive to salt. Among various strains of Propionibateria, *P. shermanii* is the most salt-tolerant strain, with critical NaCl concentration 1.15 M (~6.7%, w/w) (Ruegg and Blanc. 1981). However, it is also to be noted that salt in Swiss cheese barely reaches this level. With the low salt content in baby Swiss cheese, Propionibacteria are not inhibited. To ensure this, the salting process is less intensive in Swiss cheese than other cheese (Frohlich-Wyder and Bachmann, 2004).

After brining, the cheese blocks are allowed to dry before packing. Some of the commonly used packing blocks for rindless block cheese include prefabricated double wound Saran^{TM} bags, heat sensitive plastic pouches, air/water resistant sheet films (Reinbold, 1972). Swiss cheese has a distinctive aging process that is very important for proper eye development. In the pre-cooling stage, the cheese blocks are held at 10 to 15°C for about 5 to 10 days (21 CFR 133.195, FDA, April 2014). During the pre cooling stage the cheese body firms and pH decreases by 0.1 - 0.2 units (Cakir and Clark, 2009). The cool room is followed by warm room treatment at ~23°C for 3 to 4 weeks approximately, where eye development takes place by the fermentation of propionic acid bacteria (explained in detail later in this section). After desirable number and size of eyes are formed, the cheese blocks are shifted to a cold room at 4°C. This step slows down the bacterial growth, along with firming the curd for easier handling and distribution purposes. Swiss and baby Swiss are typically marketed soon after the 60-day aging period.

Two Major Fermentations

Swiss cheese manufacture involves two major fermentations. The first step is conversion of lactose to lactic acid by lactic-acid bacteria, namely *S. thermophilus* and *L.*

helviticus. S. *thermophilus* is the primary producer of lactic acid (Steffen at al., 1993). These two organisms are known to act synergistically such that the growth of one organism promotes he growth of the other (Hutkins, 2006). These cultures aid in gradual development of acid from the time of addition until curds are formed. However, the actual fermentation happens after the curds are out of the vat while the curds maintain an internal temperature of 35°C for several hours (Hutkins, 2006). It is to be noted that the temperature optimum for maximal growth rates of these bacteria is 42 to 45°C, while the maximal acid production by these bacteria in milk requires a mean temperature of 42.7°C (*S. thermophiles)* and 44°C (*L. helviticus)* (Martley, 1983). In the first several hours of fermentation, S. *thermophilus* utilizes the major part of available lactose and convers it into glucose and galactose by the enzyme ß-galactosidase (Hutkins, 2006). Glucose alone is further catabolized to lactic acid. Among the two, S. *thermophilus* starts its action first partly because it is usually added at a higher concentartion than is L. *helviticus* (10:1; Hutkins, 2006). After about 8 to 12 hours, L. *helveticus* competes for remaining lactose, which is subsequently catabolized. It also catabolizes the released galactose, relatively slowly (Cakir and Clark, 2009) so that all the carbohydrates in curd are metabolized after about 18 to 24 hours (Hutkins, 2006). *L. helveticus* controls pH by acting as a secondary acid-producer and plays an important role in contributing to proteolysis and producing flavor compounds (Palma et al., 1987). The main end products of this fermentation from initial stage until before brining are D- and/or L-lactic acid (Salminen and Wright, 1998). The pH of the cheese after 24 hours is ~5.2, provided there was the right amount of lactose after the cooking process and all of the lactose was fermented towards the end of fermentation (Hutkins, 2006).

The second important fermentation that takes place after the lactic fermentation is conversion of lactate to propionate, acetate, and carbon dioxide by propionic acid bacteria (Steffen et al., 1993). Propionic acid fermentation is usually initiated in the warm room ripening (25 ± 5°C) after a week of cold room ripening (10°C). The warm room ripening is usually 21 to 28 days long approximately. *Propionibacterium freudenrichii* subsp. *shermanii* is the main culture involved in the warm room ripening process. Obtaining good quality cheese depends mainly on S. *thermophilus* and Propionibacteria (Reinbold, 1972). Selected species of *P. shermanii* are used to ripen cheese with propionic acid fermentation in order to achieve desirable eyes and nutty flavor (Frohlich-Wyder M.T., Bachmann H.P., 2004). Propionibacteria are neutrophiles (organisms that grow in neutral pH environment) and salt-sensitive (as mentioned earlier) and thus low pH and high salt concentration are inhibitory to these organisms. *P. shermanii* generally prefers anaerobic environment for growth (Hutkins, 2010). Their optimal growth temperature ranges from 28 to 30°C (Vorobjeva, 1999). Propioniacteria are capable of metabolizing lactate into propionic acid, acetic acid, and carbon dioxide; therefore, the metabolites released from this fermentation contribute to typical Swiss cheese flavor, while CO_2 leads to the formation of eyes. For the development of free fatty acids and amino acids necessary for typical Swiss cheese flavor, Ji et al. (2004) recommended to keep the cheese in the warm room for at least 3 weeks. Apart from volatile and other non-volatile components, both amount of acid produced during cheese making and ripening temperatures in warm room play a major role in flavor development (Lawlor et al., 2003).

Flavor and Fatty Acid Profile of Swiss Cheese

A wide range of fatty acids has been reported in ruminant fats; approximately 400 different fatty acids have been detected in bovine milk (Christie, 1995). Lipolysis refers to breakdown of milk fat of cheese milk during ripening (Wilkinson, 2007). Lipolysis is considered one of the important biochemical reactions that take place during cheese ripening, which also helps in the formation of cheese flavor. Levels of lipolysis vary in different cheeses. Blue cheese exhibits highest levels of lipolysis (>100 mg of free fatty acids (FFA)/g of fat). Contrastingly, Cheddar and Swiss cheeses have intermediate levels of lipolysis (2 to 5 and 10 to 13 mg of FFA/g of fat, respectively)(Woo et al., 1984, Chamba and Perre' ard. 2002, Hickey et al., 2006). *Propionibacterium freudenrichii* subsp. *shermanii* is considered the major species in Swiss cheese lipolysis (Chamba and Perre'ard. 2002). Thierry et al. (2005) showed that the concentrations of FFA increased five times in cheese inoculated with *P. freudenreichii* when compared with the cheese without this bacteria. During the warm room ripening, fatty acids such as such as butyric acid ($C_{4:0}$), palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$) and linolenic ($C_{18:3}$) are known to be released significantly more than any other fatty acids (Lopez et al., 2006). Short and medium-chain fatty acids (C_4 to C_{10}) mainly contribute to acid taste of the cheese (Fox et al., 2000).

Typical Swiss cheese has a distinct nutty, sweet flavor because of interaction of FFA, peptides, amino acids, and carbonyls (Griffith and Hammond, 1989). However, the unique Swiss cheese flavor is mainly due to presence of acetic acid, propionic acid, n-caproic acid, isobutyric and, isovaleric acid (Bosset et al., 1993). Isovaleric acid in particular has rancid, cheesy, sweaty and putrid odour that could contribute to a highly-ripened cheese aroma (Yvon et al., 2001). Based on specification of the U. S. grading system for U. S. Grade A, the

overall flavor of Swiss cheese should be pleasant, with desirable characteristic flavor consistent with age and free of off-flavors (USDA, Agricultural marketing service dairy program, 2001). According to these standards, U. S. Grade A Swiss cheese should be devoid of even slight notes of undesirable flavors, namely metallic, sour, bitter, rancid, and acidic. However, these flavor notes are allowed in U. S. Grade C Swiss cheese at controlled level of flavor perceptions (USDA, Agricultural marketing service dairy program, 2001). Rancid (soapy-notes) in Swiss cheese is mainly from perception of butyric acid or short-chain volatile fatty acid (Cakir and Clark, 2009). In a descriptive analysis conducted by Liggett et al. (2008), butyric acid notes and cowy aromas were found only in commercial cheeses during training but not in any of their experimental cheeses (including 10 Swiss cheese, 4 baby Swiss cheeses and one Swiss Emmentaler). The FFA composition of Swiss cheese compared with a few rancid cheeses, namely Romano and Provolone, is provided in Table 1.2. It is to be noted that the butyric acid content is about 10 times more in Romano and 4.6 times more in Provolone compared to that of Swiss cheese (Table 1.2).

Factors Affecting Composition and Eye Development of Swiss Cheese

Eye development occurs during the warm room ripening at temperature ~22°C as a result of production of CO_2 by *P. shermanii* by fermenting lactic acid. According to U. S. Grade-A standards, Swiss cheese should possess well-developed round or slightly oval-shaped eyes that are relatively uniform in size and distribution with the majority of eyes 3/8 to 13/16 inch in diameter (USDA, 2001). Eye formation in Swiss cheese depends upon appropriate physiochemical and mechanical properties. Steffen et al. (1993) mentioned that the eye formation depends upon time and intensity of CO_2 production, areas of future eye formation, body, and texture of cheese. Cakir and Clark (2009) stated that for ideal cheese,

eyes should be distributed in the center. A pliable and elastic curd mass is required for accommodation of eyes in the cheese network. However, if the structure of cheese is too moist or too rigid, the cheese network cannot withstand the pressure and could lead to breaking of eyes and forming splits. The elasticity of the cheese usually depends upon protein density, temperature, and physiochemistry of cheese (Johnson, 2001; Mocquot, 1979). Lawrence et al. (1984) discussed the factors that determine the flavor and eye formation during cheese making in a flow chart (Figure 1.2).

Table 1.2: Typical free fatty acid (FFA; mg/kg cheese) composition of Swiss and other cheese (Woo et al., 1984)

FFA	Swiss cheese	Provolone	Romano
$C_{4:0}$	170	782	1756
$C_{6:0}$	90	308	843
$C_{8:0}$	45	81	328
$C_{10:0}$	122	172	942
$C_{12:0}$	208	122	428
$C_{14:0}$	311	120	448
$C_{16:0}$	1904	199	785
$C_{18:0}$	1427	334*	1224*
Total	4277	2118	6754

* = Value includes $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$.

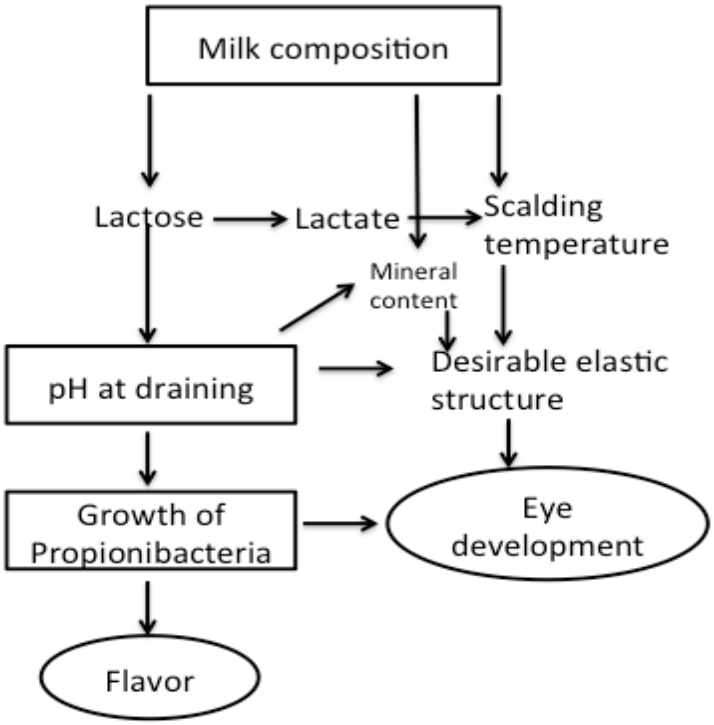

Figure 1.2: Factors influencing flavor and eye formation during cheese making (adapted and modified from Lawrence et al., 1984)

Late-blowing in Swiss Cheese

Eye formation is considered desirable when it is formed by CO_2 produced as a result of fermentation by starter (*P. shermanii*). However, contamination by non-starter bacteria such as *Clostridium tyrobutyricum* could lead to the production of H_2 gas in addition to CO_2, butyric acid, and acetic acid by utilizing lactate substrate (Frohlich-Wyder and Bachmann, 2007). The H_2 gas produced is insoluble in cheese body compared with CO_2 and thus leads to production of sudden large openings in the cheese body. Too much gas (Co_2 and H_2) produced at once elevates the gas pressure higher than the ability of curd to withstand, leading to blowing or exploding in cheese body (Hutkins, 2006). This causes variation on eye size (forming too large or to numerous eyes), and the resultant cheese body has eyes that are

usually split and/or cracked, leading to splits or the late-blowing defect. Unforeseen gas formation in the cheese, such as CO_2, H_2, or H_2S is usually associated with unclean and strong atypical off-flavors (Frohlich-Wyder and Bachmann, 2007, Cakir and Clark, 2009). Another theory by Hettinga et al. (1974) states that the Propionibacteria strains that are active at low temperatures could lead to the late-fermentation forming eyes after the cheese is shifted from warm room to cold room. The temperature difference when the cheese is shifted from warm room to cold room increases the rigidity, resulting in brittle cheese body. Any further gas production by non-starter Propionibacteria could also contribute to slit formation because the cheese body is incapable of accommodating gas bubbles.

Indeed, various researchers have studied the split defect and mechanisms of split formation by hypothesizing various theories. These include the elasticity of cheese and contribution of proteolysis (Johnson, 2001); secondary fermentation, where starter or non-starter bacteria produce gas during and/or after the normal warm room ripening, causing splitting and irregular eyes (Park at al., 1967, Hettinga et al., 1974, Hutkins, 2006, Frohlich-Wyder and Bachmann, 2007); presence of *Clostridium tyrobutyricum* spores (Dasagupta and Hull, 1989; Steffen et al., 1993; Frohlich-Wyder and Bachmann, 2007); and low quality feed with high spores acting as source of Clostridial contamination in milk and cow environment (Dasagupta and Hull, 1989; Houck et al., 2007). Although several explored the major causes of late-blowing in Swiss-type cheeses, so far there is no consistent, convincing evidence on the mechanism of split formation and how it can be avoided.

Corn Distillers' Grains

Corn is a major crop grown in the Midwest region of the U.S. Two types of corn milling processes exist currently, each of which results in a variety of products. Dry milling

produces distillers' grains with solubles, while wet milling produces corn gluten feed (Erickson et al., 2005). In this current study, as our interest is mainly in distillers' grains (**DG**), the production of DG along with ethanol and other co-products through corn dry-grind process is shown in a flowchart (Figure 1.3) and summarized below.

Ethanol production from corn involves fermentation by the yeast *Saccharomyces cerevisiae* (U.S. Grains Council, 2010). After fermentation and distillation of ethanol, whole stillage (water and solids) is produced as a co-product. Through centrifugation, whole stillage is separated into thin stillage and coarse solids or wet distillers' grains (**WDG**, ~35% DM). These WDG are further dried in a rotary drier to form dried distillers' grains (**DDG**). The mixture of syrup (30% DM), obtained after evaporation of thin stillage, and DDG results in the production of dried distillers' grains with solubles (**DDGS**, ~90% DM). With the U.S. being currently the world's largest ethanol producer, its production has increased from 0.7 million cubic meter in 1980 to 18 million cubic meter in 2006 which then increased to 10.2 billion gallons in 2009, consequently increasing the production of its by products as well.

Most ethanol co-products are now available as distillers' grains with solubles (**DGS**), and it is a major by-product of ethanol production that is currently being fed to dairy cattle (Schingoethe et al., 2009). Our current interest is specifically in dried DGS (DDGS) because of its availability in the Midwest. In 2010, the estimated supply of U.S. DDGS was about 33.3 million metric tons (Hoffman and Baker, 2010). The composition of DDGS has been of great interest in the area of animal science, as it is being widely used as a feed ingredient to livestock. The basic composition of DDGS reported by three researchers is provided in Table 1.3.

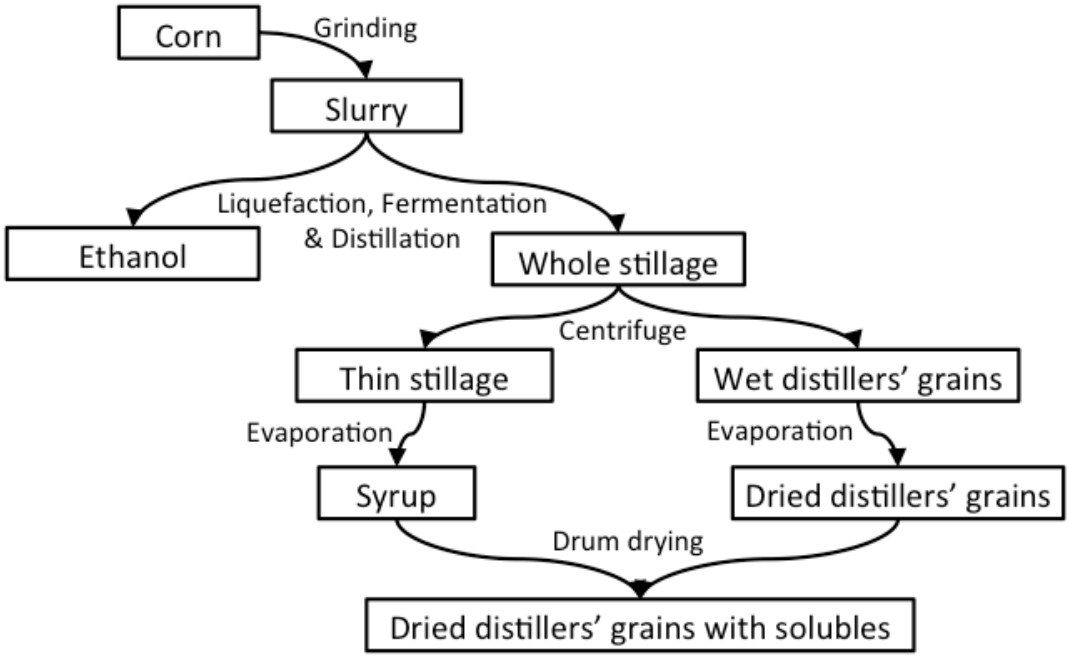

Figure 1.3: Dried distillers' grains with solubles production from corn by dry-grind basis along with its co-products

According to Taheripour et al. (2010), 16% of corn-based ethanol plants revenue comes from DDGS. The use of DDGS in the livestock industry, particularly feeding to dairy cows, is increasing because of it high protein and energy content (Schingoethe et al., 2009, Mjoun et al., 2010)). In fact, the practice of feeding distillers' grains to cattle has been in place since 100 years ago (Loosli et al., 1952). Milk production tends to be higher when fed with DDGS compared with soybean meal-based control diets (Kleinschmit et al., 2006). Anderson et al. (2006) showed feeding 20% of diet dry matter of wet or dried DGS replaces 25% of the corn and 87% of the soybean meal.

Table 1.3: Composition of DDGS (% dry basis) from three sources

	Spiehs et al., 2002	Belyea et al., 2004	Kim et al., 2008
Moisture	11.1%	NA[1]	11.2%
Crude protein	30.2%	31.3%	24.9%
Lipids	10.9%	11.9%	14.5%
Crude fiber	8.8%	10.2%	NA
Starch	NA	5.1%	5.2%
Acid detergent fiber	16.2%	17.2%	NA
Ash	5.8%	4.6%	4.5%

[1]NA= Not available

There are various concerns associated with feeding DDGS to dairy cattle. Recently, the impact of feeding distillers' grains to dairy herds has led to questions about oxidized off flavors in milk (Testroet et al., 2015; in press at J. of Dairy Sci.) and late blowing in cheese (Houck, et al., 2007). However, Testroet et al. (2015) concluded that feeding cows with 10% and 25% DDGS did not induce any apparent oxidation or spontaneous oxidized flavor in milk. Indeed, the objective of our current study focuses on the investigating the impacts of feeding DDGS on the quality of baby Swiss cheese.

Clostridium tyrobutyricum spores, sometimes associated with late-blowing defect (Klijn et al. 1995), are known to end up in the milk from poor quality silage-fed livestock

(Buch Kristensen, 1999). Another important route through which spores could access the milk is through dung remaining on the teats during the milking process. Dasagupta and Hull (1989) reported a positive correlation between the concentration of *C. tyrobutyricum* and other anaerobic spores in feedstocks (silage, brewers grain, potatoes), cow dung, and milk. *Clostridium* spp. ending up in the raw milk is capable of surviving the pasteurization process (Ingham et al., 1998). Klijn et al. (1995) showed that pure cultures of *C. tyrobutyricum* had the ability to produce the late blowing defect in cheese. Presence of splits in the cheese body makes the cheese inappropriate for use on the mechanical high speed slicing equipment causing considerable loss of product (Klijn et al., 1995, White et al., 2003). Reinbold, (1972) described that this defect caused because of unwanted or excessive gas production that appears as splits or cracks in cheese body, leading to downgrading of cheese.

Antibiotic Usage in Ethanol Production Process

During the ethanol fermentation process, antibiotics have been used to control unwanted bacteria for many years (Juranek and Duquette, 2007). Antibiotics kill lactic acid bacteria that compete with yeast for glucose and other nutrients during the yeast (*Saccharomyces cerevisiae*) fermentation. Currently, the only antibiotic that has been provided with "no objection letter" from FDA (Nov 16, 1993) for use in the ethanol is virginiamycin. Virginiamycin is effective against seven strains of Lactobacilli during alcoholic fermentation, but is not effective against *L. rhamnosus*, *L. paracasei* and *L. plantarum* (Hynes et al. 1997). The FDA's Center for Veterinary Medicine (CVM) sets the maximal level of distillers' by-products' residual level of virginiamycin of 0.2 to 0.5 ppm. About 2 to 6 ppm of virginiamycin is allowed to be added during the fermentation process (National Grain and Feed Association, 2009). The U.S. Food and Drug Administration

approved level of virginiamycin in cattle feed is 100 to 340 milligrams per head per day. Virginiamycin has been shown to improve milk yield in lactating dairy cattle (Clayton et. al., 1999). Current FDA recommendations discourage the use of antibiotics for improving production parameters in livestock, and, instead, the FDA recommends "judicious" use of antibiotics in livestock to help prevent the development of antibiotic-resistant microbes (FDA, 2014).

Because antibiotics are routinely used in the production of ethanol and, consequently, the production of DDGS, feeding DDGS could potentially result in inadvertent feeding of antibiotics to ruminant animals. A study conducted by FDA in 2012 reported that 3 out of 28 (10.7%) samples of DG had detectable antibiotic residues. Out of those 3 samples containing antibiotic residues, one sample contained 0.16 mg/kg of virginiamycin M1 residue; a second sample contained 0.15 mg/kg of erythromycin; a third sample contained approximately 0.15 mg/kg of virginiamycin M1 residue and 0.24 mg/kg of penicillin G (Fairfield, FDA, 2012). Determination of the presence or lack of antibiotics in distillers' grains is important, but it is additionally important to determine the biological activity of these antibiotics. Recently, a study conducted by the Paulus Compart et al. (2013) quantified the concentration of various antibiotic residues including erythromycin, penicillin G, tetracycline, virginiamycin M1, tylosin in distillers' grains (wet: 79 samples, dry: 80 samples) and tested whether they were biologically active by using liquid chromatography and mass spectrometry. Antibiotic residues were found to be very low and less than the maximal concentrations approved by FDA in feed for food-producing animals. However, there are chances of toxic effects during combined action of antibiotics present. For instance, macrolide antibiotics such as erythromycin and virginiamycin have the chance of inducing monensin toxicity by delaying

its clearance in the liver when supplemented (Basaraba et al., 1999). Basaraba et al. (1999) reported a case where monensin caused death of cattle fed DDG containing macrolide antibiotic (50 to 1500 mg/kg) combination of erythromycin, clarithromycin, and others). Any residual antibiotics remaining in DDGS, however, could be detrimental in animal feeding programs because misuse or over-dosing of antibiotics could lead to development of antibiotic resistance in bacteria. Further, it is very important that dairy cows are not fed anything that could contaminate or pose long-term detrimental effects on cows, rumen microflora, or milk.

Various studies mentioned above recommend using DDGS in the diet of dairy herds for their role in augmenting milk yield and protein. However, there are also several concerns associated with feeding distillers' grains (Schingoethe et al., 2009). Though there are researchers (Dasagupta and Hull, 1989, Houck et al., 2007) who hypothesized the association between feed (e.g. distillers' grains, silage) and late-blowing defect in cheese, there is still no consensus on the major cause of the defect and how to avoid it. The present study was designed to investigate the effects of feeding DDGS to dairy cattle on the occurrence of late-blowing defect in baby Swiss cheese. This study also investigated if the DDGS used for the feeding trails had any antibiotic residues, as it is widely accepted that inadvertent feeding of antibiotics to dairy cows through DDGS could have important implications for the dairy industry at large, particularly if any antibiotics in DDGS are transferred into milk.

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CHAPTER 2. DRIED DISTILLERS' GRAINS WITH SOLUBLES (DDGS) ARE NOT TO BLAME FOR LOW-QUALITY BABY SWISS CHEESE

A paper to be submitted to *The Journal of Dairy Science*

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Abstract

Late blowing in Swiss cheese, a result of unwanted gas production during ripening, is unacceptable to consumers, and causes economic loss to manufacturers. Cheese processors have raised concerns that feeding dried distillers' grains with soluble (DDGS) to cows leads to this defect, in part, because of *Clostridial* spores. In this study, the effect of feeding DDGS to lactating dairy cows on composition and quality of milk and baby Swiss cheese was examined. Thirty healthy multiparous and mid-lactation Holstein cows were assigned randomly to one of three dietary treatment groups (10 cows per treatment group): (1) total mixed ration (TMR) with no DDGS, (2) TMR with 10% DDGS by dietary DM, and (3) TMR with 20% DDGS by dietary DM in a 3 × 3 Latin square with repeated measures. One complete milking from all cows within a treatment was collected and pooled for cheese-making trials, twice within each month of the three-month study. Additionally, individual milk samples from three milkings of a day were collected weekly, and proximate analysis was carried out on pooled individual milk samples. Milk used for cheese production was standardized to 0.88 fat:protein, and pasteurized before making baby Swiss cheese. The ~3.5

kg cheese blocks were vacuum packed and allowed to ripen (10^0C, 7 days; then 22^0C, 21 days), then cooled (4^0C, 60 days). Milk, cheese, TMR, DDGS and manure were analyzed for gas formation by Clostridial spores. Within 48 hours incubation in modified reinforced clostridium lactate medium, tubes containing milk, cheese, TMR, or manure showed gas formation. Conversely, DDGS used in our study was not a source of gas-producing spores. Feeding 10% and 20% DDGS decreased milk fat content ($P < 0.0001$) and increased the solids nonfat ($P < 0.005$), protein ($P < 0.05$), and lactose concentration of milk ($P < 0.05$) when compared with the milk from cows fed the control diet. After 60 days ripening, baby Swiss cheese had typical propionic acid Swiss cheese aroma. Regardless of diet treatment, pinholes, slits, and cracks were seen throughout most cheeses. Feeding of DDGS increased the amount of long-chain unsaturated fatty acids and decreased short-chain and most medium-chain fatty acids in the baby Swiss cheese. Although feeding cows with DDGS, modified milk composition and subsequently the cheese composition, DDGS could not be blamed as a source for gas-producing spores or for quality defects in Swiss cheese, but, rather, the gas-producing spores likely originate from the cow herself or the environment.

Key words: ethanol, eyes, Clostridium, spores, total mixed ration

Introduction

The late blowing defect in Swiss-style cheese varieties can be described as appearance of undesirable slits, cracks or blown areas in the cut cheese. Late-blown cheese is unacceptable to consumers and high-speed slicing operations; because of which it cannot be sold at full value, which leads to economic losses to cheese producers. Reports on late-blowing in cheeses have raised concerns about feeding distillers' grains to dairy herds contributes to the late-blowing in cheese (Houck et al., 2007).

Corn is a major crop grown in the Midwest region of the United States. With the US being currently the world's largest ethanol producer, its production has increased from 0.7 million cubic meter in 1980 to 18 million cubic meter in 2006, which then increased to 10.2 billion gallons in 2009, consequently increasing the production of its by products as well. Two types of corn milling processes exist currently, each of which results in a variety of products. Dry milling produces distillers' grains with solubles while wet milling produces corn gluten feed (Erickson et al., 2005). In this current study, as our interest is mainly on distillers' grains (**DG**), the production of DG along with ethanol and other co-products through corn dry-grind process is shown in a flowchart summarized in Fig 1.3 in Literature review section.

Ethanol production from corn involves fermentation by yeast (*Saccharomyces cerevisiae;* U. S. Grains Council, 2010). After fermentation and distillation of ethanol, whole stillage (water and solids) is produced as a co-product. Through centrifugation, whole stillage is separated into thin stillage and coarse solids or wet distillers' grains (**WDG**, ~35% DM). These WDG are further dried in a rotary drier to form dried distillers' grains (**DDG**). The mixture of syrup (30% DM), obtained after evaporation of thin stillage, and DDG results in the production of dried distillers' grains with solubles (**DDGS**, ~90% DM).

Eye formation in Swiss cheese is considered desirable when it is formed by carbon dioxide produced as a result of fermentation by starter (Propionibacterium *freudenrichii* subsp. *shermanii*). However, contamination by non-starter bacteria such as Clostridium *tyrobutyricum* could lead to the production of H_2 gas in addition to carbon dioxide, butyric acid, and acetic acid by utilizing lactate substrate (Frohlich-Wyder and Bachmann, 2007). This hydrogen gas produced is insoluble in cheese body compared with carbon dioxide and

thus leading to production of unforeseen large openings in cheese body. Too much of gas (CO_2 + H_2) produced at once elevates the gas pressure higher than the ability of curd could withstand leading to blowing or exploding in cheese body (Hutkins, 2006). This what causes variation on eye size (forming too large, too small, or too numerous eyes) and the resultant cheese body has eyes that are usually split and/or cracked, leading to split defect or late-blowing defect. Unforeseen gas formation in the cheese, such as CO_2, H_2, or H_2S is usually associated with unclean and strong atypical off-flavors (Frohlich-Wyder and Bachmann, 2007, Cakir and Clark, 2009). Another theory by Hettinga et al., (1974) linked Propionibacterium strains that are active at low temperatures to the late-fermentation forming eyes after the cheese is shifted from warm room to cold room. The temperature difference when the cheese is shifted from warm room to cold room increases the rigidity, resulting in a brittle cheese body. Any further gas production by non-starter Propionibacteria in the cold room could also contribute to slit formation because the cheese body is incapable of accommodating gas bubbles.

Indeed, various researchers have studied about split defect and mechanism of split formation by hypothesizing different theories. These include the elasticity of cheese and contribution of proteolysis (Johnson, 2001); secondary fermentation, where starter or non-starter bacteria produce gas during and/or after the normal warm room ripening, causing splitting and irregular eyes (Park at al., 1967, Hettinga et al., 1974, Hutkins, 2006, Frohlich-Wyder and Bachmann, 2007); presence of *Clostridium tyrobutyricum* spores (Dasagupta and Hull, 1989; Steffen et al., 1993; Frohlich-Wyder and Bachmann, 2007); low quality feed with high CLostridial spore count acting as source of contamination in milk and cow environment (Dasagupta and Hull, 1989; Houck et al., 2007). Although several researchers were involved

in exploring the major causes of defect, so far there is no convincing evidence on the mechanism of split formation and how it can be avoided.

With ethanol production being a major industry in the Midwest, utilization of protein-rich DDGS in animal feeding is inevitable and generally considered economical. Consequently, it is of high priority to processors in the Midwest that we understand the effects of DDGS inclusion in the diet of dairy cows on quality markers of milk as they contribute to cheese production. The objective of this research is to investigate the impact of feeding diets with three levels of DDGS (0%, 10% and 20% of the total mixed ration) to dairy cows on milk and cheese composition, eye formation and micro flora.

Materials and Methods

Experimental design

Feeding trials were conducted at the Iowa State University dairy farm (Ames, IA) between July and October 2013. Thirty healthy multiparous and mid-lactation Holstein cows were selected and stratified by days in milk (DIM) and parity to one of the three dietary treatment groups with ten cows in each treatment group in a 3×3 Latin Square with repeated measures. The diets were formulated to be isonitrogenous (16.5% crude protein) and isoenergetic. The treatments included (1) total mixed ration (TMR) with no DDGS, (2) TMR with 10% DDGS by dietary DM, and (3) TMR with 20% DDGS. The TMR and DDGS composition are reported separately in detail (Testroet et al., 2015; in submitted, J. of Dairy Sci). Cows were trained to use Calan® gates (American Calan Inc., Northwood, NH) where each cow had *ad libitum* access to food and water. After each treatment period, the cows were switched to the next diet, such that each cow served as her own control for a total of

three diet treatments. To minimize carryover effects, the first 14 days of each 28-day period were not included in the analyses. Milk from all the three milkings from each cow were collected and pooled to represent an entire milking from that individual cow every week on Tuesday and analyzed on Wednesday.

Proximate analysis

The milk from each cow was collected throughout one entire milking by a Boumatic milking system (Boumatic, Madison, WI), then poured into blue 60 mL snap cap bottles (Fischer Scientific, Pittsburgh, PA), transported to the laboratory within 30 minutes of collection, and refrigerated (~4°C). Proximate analyses (percent lactose, fat, and protein) then were performed on the individual milk samples in duplicate by using a Lacticheck-01 RapiRead Milk Analyzer (Page & Pedersen Intl. Ltd., Hopkinton, MA); pH was measured using an Accumet® Basic AB15 (Fisher Scientific Inc, PA). The chilled milk samples were inverted gently inside the blue snap cap bottles to homogenize any separated cream on the cap of the bottle and tempered to room temperature (22 ± 2°C) before analyzing. Introduction of air bubbles was avoided while mixing the sample as it could introduce error into the readings from the Lacticheck™ milk analyzer.

Milk collection for cheese making

Milk from one complete milking was collected twice during each 28-day period (periods 1, 2 and 3) from each treatment group (0%, 10%, 20% DDGS). For cheese-making, the morning milking (6.30 am) was collected. Typically, milk from 2 groups of 10 cows each on Friday and 1 group of 10 cows on Saturday were collected and pooled for use in cheese making trials. The milk cans were washed with automatically diluted Ecolab® Oasis

Enforce (St. Paul, MN) and sanitized with automatically diluted Ecolab® Mikroklene® (St. Paul, MN) sanitizer or manually diluted 100 to 150 ppm Clorox® (Oakland, CA) solution. Milk was collected by using sanitized dump buckets and transferred into sanitized, labeled aluminum milk cans. The milk cans were then transported to Center for Crops Utilization Research (CCUR) pilot plant in the Food Sciences Building on Iowa State University campus by 8.00 am. The milk cans were immediately placed in the walk-in cooler at 4^0C until further processing.

In the meantime, those who collected milk at the dairy farm were requested to take a shower or change to clean clothes before participation in cheese making to minimize additional external contamination of milk to be used for cheese production. The exteriors of milk cans were rinsed with cold water before filtration (through the cheesecloth settled on metal mesh sieve) and weighing (into pre-weighed, tarred, empty aluminum cans).

Milk samples from cans were transferred into sanitized cans very gently to minimize frothing and agitation. The weighed milk was mixed before the sample was drawn for proximate analysis as previously described. Measured percent fat and protein were used to standardize milk to the target fat:protein ratio (0.88±0.05). If the fat:protein ratio was not 0.88±0.05, the milk was separated and standardized, and cream or skim were added to raise or lower the ratio, respectively. Milk was separated using a Type LWA 205 Westfalia Separator (219 rpm in 2.5 dial setting, Dusseldorf, Germany) from the Center for Crops Utilization pilot plant of Iowa State University.

Subsequently, milk was HTST pasteurized with a UHT/HTST Electric model 25HV Hybrid pasteurizer (MicroThermics®, Releigh, NC). Prior to start-up, the pasteurizer was rinsed with cold water, soap, spore cleanser, and sanitizer before the start of each cheese

make. The pasteurization temperature was set at 73.5°C for 15 sec (flow rate 4 L/min). Upon exit from the pasteurizer (~37°C), milk was filled into one of two cheese vats and cooled down to 33°C by running cold water in the jacketed vat, with gentle agitation of the milk.

Baby Swiss cheese was made using cultures namely *Streptococcus thermophilus SSC 17 (Chr Hansen, Milwaukee, WI)*, *Lactobacillus helveticus (DuPont* ^{TM} Danisco®, Madison, WI), *Propionibacterium freudenrichii* subsp. *shermanii* (Chr Hansen PS-1, Milwaukee, WI). Coagulant (DSM Maxiren®, The Neatherlands) was diluted with cold water to a ratio of 1:40 and added at 4mL/45 kg of milk and the cheese curd was allowed to set for about 25 – 30 min. The curd was tested for firmness and manually cut at slow-speed. About 25% of vat volume of whey was initially removed followed by constant stirring and addition of water (3 to 5% of the vat volume) at 33°C. Gradually, the curds were cooked by increasing the temperature to 40°C and then to 46°C over a 10-min period by adding steam to the sides of the vat. In the mean time, warm water (~10 % of the vat volume) was added at 44°C to facilitate the rise in temperature of the cheese to 46°C. The cook speed was decreased gradually by switching off the steam to reach the final target temperature at 47.8°C at which the curds were allowed for a 42-minute incubation. After the incubation step, the target pH was 6.4 and enough whey was removed to cover the curds (~3 inches). Time, temperature, and pH were analyzed constantly at various time-points of the cheese making process.

After removing all free whey, the cheese curds were collected into perforated cheese molds made by drilling holes in 5-gallon plastic storage basin containers. Blocks were pressed under whey by using the same style plastic basin filled with water (hydraulic weight), which weighed about 16 kg for about 15 min. Next, the whey was drained completely and the cheese block was pressed for about 5 hr. The pH of the curd was

measured after 2 hr and the curd block was flipped. The press was removed, and cheese was fermented an additional 8 – 10 hr at 28°C. The length of time taken for pressing was based on the time required for the pH of the cheese to drop from 6.4 to 5.25. Brining was carried out in saturated brine containing 23% NaCl and 76 mL of 0.2% $CaCl_2$, for about 12 hr. Cheese blocks were vacuum-packed in clear vacuum seal bags (Fisher Scientific Inc, Pittsburgh, PA) by using gas flash vacuum packing machine (Koch Equipment LLC©, Kansas City, MO). Cheeses were stored at 10±5°C for 7 days (Pre-cool), 22 5°C for 21 days (warm room), and 4°C for 60 days (cold room). Cheeses were analyzed for composition, quality, and spores after 60 days in the cold room. The cheeses were cut into pieces for analysis, and pictures were taken.

Staff at the Agricultural Utilization Research Institute (AURI; Marshal, MN) carried out the cheese proximate analysis. Standard methods were followed for measuring different components of cheese (moisture: AOAC 926.08, protein: AOAC 2001.14, ash: AOAC 935.42, fat: AOAC 933.05, calcium: AOAC 991.25, sodium: AOAC 991.25, and Tbars: J. Am. Oil Chem. Soc. 1960, 37, 44-48). AURI staff also carried out the cheese fatty acid profile analysis (AOCS Ce 2 – 66, Ce 1j – 07).

Spore testing on DDGS, TMR, milk, cheese and manure

DDGS, TMR (with and without DDGS), milk, baby Swiss cheese, and manure were evaluated for the presence of gas-producing spores by observing gas formation in 50 mm Durham tubes (Fischer Scientific, Pittsburgh, PA) placed in 20 mL glass test tubes containing modified reinforced clostridium media with lactate (RCM–lactate, Dasagupta and Hull, 1989) within 48 hr. The modified RCM-lactate media was made using beef extract (10 g), yeast extract (3 g) (BD chemicals, Sparks MD), sodium chloride (5 g), L-cysteine (0.5 g),

soluble starch (1 g), (Fischer Scientific, New Jersey NJ), tryptone (10 g), sodium lactate syrup (10 mL), sodium acetate.$3H_2O$ (8 g) (Sigma Aldrich, St. Louis MO), and Agar (2 g) (BD chemicals, Sparks MD) in 1 L distilled water, and autoclaved.

Twenty-five grams of sample was mixed in 225 mL of 0.1% peptone water by water using a stomacher 400C (Seward®, Daive FL) for 30 sec. From the stomached sample, about 10 to 20 mL of liquid sample was transferred in a 30 mL glass test tube and heated to 80°C for 10 min in a water bath and cooled immediately after 10 min to kill most of the vegetative cells and enrich spores. One mL of the heat shocked sample was transferred to a clean, autoclaved test tube with 9 mL modified RCM lactate broth containing a Durham tube and stored at 35°C in anaerobic conditions by using GasPak (BD chemicals, Sparks, MD). The experiment was conducted in duplicate. Gas formation in the samples was observed by checking tubes for the presence of gas after 24 to 48 hr.

Statistical analysis

During the feeding trails, the cows that were diagnosed with mastitis or any other sickness were removed from the study. The study started with 30 cows in period 1 but decreased to 25 by the end of the study. All statistical analysis was carried out using SAS 9.3 (Cary, NC). A mixed model was used with diet, period, parity, and diet by trial interaction as fixed effects, and days in milk as covariate. Analysis was done as repeated measures with date as the repeated statement and cow nested within diet and trial as the subject. The first two weeks of each period was considered as an acclimation period and were not included in the statistical analysis. Tukey-Kramer multiple pairwise comparison adjustment was used to identify significant differences at $(P < 0.05)$

Results and Discussion

Milk data analysis

After excluding the first two weeks of data from each period, milk samples were collected from each cow for totally 10 times. DDGS feeding did not have any effect on pH ($P > 0.05$), but period had an effect on pH. The likely explanation for the period effect could be calibration errors in the pH meter. Milk protein%, and SNF% increased significantly ($P < 0.05$) with 20% dietary inclusion of DDGS. These findings were supported by our previous research (Testroet et al., 2015; in press J. of Dairy Sci.) where the treatments included 10 and 25% DDGS in TMR. The same trend of increase in protein percent was not observed by previous studies (Anderson et al., 2006, Kleinschmit et al., 2006) when fed with 20% DDGS. However in these studies, the milk yield was increased by 20% DDGS treatment, which could have diluted in the protein and decreased the protein concentration. In our study, the protein yield remained unaffected by the DDGS treatments with a non-significant decrease in milk yield. This could have concentrated the protein in the milk, which lead to significantly higher protein% in milk from 20% DDGS treatment.

The milk from cows fed 10% and 20% DDGS had significantly lower fat% ($P < 0.05$) compared to the milk from cows fed with 0% DDGS. The same pattern of depression in milk fat % and daily milk fat yield was not observed in the previous studies that fed DDGS to the dairy herds (Anderson et al., 2006, Kleinschmit et al., 2006). DDGS is known to be highly energetic partly due to its amount of fat content which is mostly unsaturated, with more than 60% linoleic acid ($C_{18:2}$) (Schingoethe et al., 2009). However, milk fat content could be highly influenced by dietary fat supplementation. Dairy nutritional experts limit the dietary inclusion of DDGS to 20% (Anderson et al., 2006) because increased unsaturated fatty in

ruminants' diet could lead to milk fat depression. Indeed, Testroet et al., (2015, in press for J. of Dairy Sci.) confirmed that feeding unsaturated dietary fats from 10% and 25 % DDGS treatment caused significant ($P< 0.05$) milk fat depression, which supported the results of our current study.

Lactose percentage of the milk increased significantly with 10% and 20% DDGS inclusion ($P <0.01$), which supports the findings of Tanaka et al, 2011 ($P < 0.05$) who observed higher lactose content in milk when fed with 20% DDGS compared with control group. The US grains council (2012) also found a non-significant ($P > 0.05$) increase in lactose content when cows were fed with 10% DDGS. The increase in lactose may be relevant with respect to eye formation, although the present study did not directly evaluate the impact of lactose on eye formation. Lactose is the primary substrate for starter cultures used in the cheese production process. Lactose is split into glucose and galactose by the starter cultures (S. *thermophilus*, L. *helveticus*), and metabolized during the maturation of cheese. Alterations in the concentration of lactose available for metabolism by these microbes may modify the maturation and subsequent properties of cheese. It is possible that excess lactose provides substrate for secondary fermentation and late blowing. Current research in our laboratory is designed specifically to investigate this hypothesis.

Eye formation in Swiss cheese depends upon appropriate physiochemical and mechanical properties. If the milk itself contained higher lactose, then it is likely that the lactose content at the start of fermentation is also high. Lactose concentration in the curd is one of the very critical checkpoints for eye development. Hutkins (2006) mentioned that it is very important that the cheese curds contain right amount of lactose after the cooking process for the final pH to reach close to 5.2. Any deviation from this pH would increase the chances

of fracturing due to excessive gas production or incidence of blind cheese (Lawrence et al., 1984). A pliable and elastic curd mass is required for accommodation of eyes in the cheese network. Contrastingly, if the structure of cheese is too moist or too rigid the cheese network cannot withstand the pressure and could lead to breaking of eyes and forming splits.

Cheese Analysis

Baby Swiss cheese standards of identity require minimum 45% fat in solids and maximum 43% moisture (ATCP, 1985). Although there was variability among individual cheeses (data not shown), all cheeses met these standards. Average fat in cheese milk was consistent among the three treatments. The lack of significant differences ($p > 0.05$) in cheese pH, moisture, fat, sodium and salt in moisture based upon diet (Table 2.2) is expected since the goal was to perform procedures consistently among batches of cheese. Since protein in cheese milk slightly increased with inclusion of DDGS, fat:protein ratio of raw milk decreased with inclusion of DDGS (data not shown). However, on average, standardized fat:protein ratio were all within 0.03 of target (0.88) among treatments.

Although no formal sensory evaluation panel was conducted on the cheese after ~90 days of aging, informal sensory evaluation by authors revealed that Baby Swiss cheeses had a typical propionic acid Swiss cheese aroma. However, more lactic acid aroma than expected in typical Baby Swiss was present in 10% and 20% when compared with the control cheese. This was likely because lactose percent was significantly higher in 10% and 20% DDGS fed cow's milk than the control milk (Table 2.1). Additionally, some cooked egg (hydrogen sulfide) aroma was apparent in the 10% and 20% DDGS cheeses. One possible explanation for this could be because spoilage organisms such as Clostridia are capable of metabolizing

available substrate lactose, leading to production of gases like hydrogen and hydrogen sulfide along with carbon dioxide (Cakir and Clark, 2009).

Eye distribution and cheese body and texture were atypical in control and treatment cheeses. Although proximate analysis revealed that all cheeses met the standard of identity for Swiss cheese (Table 2.2), all cheeses felt more soft and moist than typical baby Swiss. Slits (Figure 2.1D), pin-holes (Figure 2.1B), cracks (Figure 2.1B), and checks (Figure 2.1C) were evident in all cheeses. Glossy, round eyes were rarely found (Figure 2.1A) beyond period one, regardless of diet treatment. Blindness was also occasionally exhibited, most typically within ¼-inch (0.635 cm) of the cheese surface. Most cheeses exhibited long longitudinal cracks or blown areas, which tended to appear along curd junctions, suggesting that pressing may have been inadequate, preventing a tight, closed body (Figure 2.1). However, although unsophisticated hydraulic pressing, rather than mechanical pressing was done, cheese body was not mechanically open; openings were atypical of gas formation.

Moisture content of our cheese was higher than those reported by White et al. (2003). Higher moisture content may lead to loss of elastic nature of cheese. White et al., (2003) noticed reduced splitting in cheese with lower moisture content. The difference in moisture may partially explain the body defects in our cheese, though moisture was within standards. Aging time is also sometimes associated with splitting of cheese. White et al., (2003) observed more splitting as the ripening time increased up to 120 days. In our study the total cheese ripening time was not more than 95 days.

On average, final cheese pH tended to be slightly lower than 5.2 (Table 2.2), perhaps because of over-active cultures resulting from low salt-to-moisture ratio (mean 1.1-1.2 % S/M). Mean calcium level (7.5-7.8 mg/g) was slightly lower than typical Swiss cheese (7.9

mg/g). Because calcium is a structural component of cheese matrix, the lower calcium may partially explain the cheese softness. Mean thiobarbituric acid reactive substances (Tbars) did not differ among cheeses (0.08 to 0.10 ppm). White et al. (2003) noticed that the factors such as pH, fat, protein, and calcium contents were not necessarily different from split and un-split cheese. In their study, they did not observe any correlation between these factors and chances of cheese being split.

Cheese fatty acid profile revealed that long chain unsaturated fatty acids increased with DDGS feeding (Table 2.3). As the goal was to maintain same fat:protein (0.88 ± 0.05) among all batches of cheese, there were no significant differences found among treatments. All short chain volatile saturated fatty acids decreased (C4, C6, C8, C10), as well as most saturated medium-chain fatty acids (C14, C16, C18). No changes were seen in C15, C17, C19 and C20, which were found only in trace amounts. The amount of C12 increased numerically with DDGS feeding, but 10% DDGS cheese had more than control and 20% DDGS cheese. All long-chain mono-unsaturated fatty acids increased numerically (9c-C14:1, 9c-C16:1, 6t-C18:1, 11t-C18:1, 9c-C18:1 and 11c-C18:1) except 9t-C18:1, 6c-C18:1 and 8c-C20:1). Poly-unsaturated fatty acids remained unchanged (9c, 12c, 15c-C18:3, 8c, 11c, 14c-C20:3 and 5c, 8c, 11c, 14c-C20:4) or increased numerically (9c, 12c-C18:2)($P>0.05$).

Spore testing

Modified RCM-lactate was prepared by substituting sodium lactate for glucose for mainly estimating the gas formation by C. *tyrobutyricum* spores. However, any gas-producing organism (for e.g. Propionibacterium) that is capable of utilizing lactates as the sole carbon source and produce gas can grow in this medium. Therefore, RCM-lactate may

not be considered as highly selective media (Wehr and Frank, 2004). However, the heat-shocking (80°C for 10 mins) step was followed in order to induce spore germination (if any) and get rid of most of vegetative cells in the samples, as the main focus of the research is to identify gas formation by spores. This would help in killing the microbes that are sensitive to heat at 80°C. Additionally, the samples were inoculated in the test tubes containing Durham's tube and allowed to incubate under strict anaerobic condition. This step would help in getting rid of any obligate aerobes present in the sample. All these steps were performed to make the medium as selective as possible and to ensure that the conditions held would support the growth of Clostridial spores over other microbes.

No gas formation by spores was observed when RCM-lactate medium was inoculated with DDGS under anaerobic conditions. The DDGS used in the feeding study was likely to be devoid of spores or microbial load. The high temperatures (up to 426.6°C) associated with the DDGS drying process likely only partially explain these results (Pfizer Animal Health, Lactrol Technical Information Pamphlet). Although Pedersen et al. (2004) did not find microorganisms in the wheat WDG samples collected directly out of the distillation column; storage in unclean environment could possible lead to increases in microbial loads. Gas formation in RCM-lactate medium was observed in all samples of baby Swiss cheese, manure and TMR under anaerobic conditions. These results suggest that TMR or the cow environment was the major source for gas forming species and not DDGS.

Herlin and Christiansson (1993) suggested that spores make their way into the milk during the milking process through manure-contaminated teats. Butyric acid bacteria spores are naturally present in the soil from which they make their way into feed and act as a source of contamination in feed, and eventually in milk (Vissers et al., 2006). One of the major

spore-forming butyric acid fermenting bacteria, C. *tyrobutyricum* is known to be propagated via feed and contaminate the milk at the farm level (Houck et al., 2007). Halligan and Fryer (1976), Dasgupta and Hull (1989), reported large number of spores in manure form the cow fed with silage containing *C. tyrobutyricm*. This species is certainly expected to be present in all the late blown cheese, perhaps a prerequisite (Klijn et al., 1995). Other Clostridial species, such as *C. beijerinckii,* could also end up in cheese, as they are common anaerobic spore formers found in milk (Klijn et al., 1995). Preventing contamination of raw milk and removing spores from contaminated milk are very important steps of avoiding economic losses because of late blowing defect. Su et al. (1999) recommended centrifugation of milk, preventing spore accumulation in brine, and decreased ripening temperatures as potential spore reducing preventive measures against defects.

Because all cheeses, including cheeses from cows not fed DDGS, exhibited body and texture defects, these findings do not implicate DDGS, but rather spores not originating from DDGS, as the likely causative agent for the defects observed. However, we cannot rule out the potential role of increased lactose concentration on baby Swiss quality defects. Research in this area is currently underway in our laboratory.

Conclusion

Feeding 20% DDGS as part of a TMR decreased percent fat and increased percent lactose and percent protein in milk. The DDGS source used for this research did not contain gas-forming spores, yet TMR, manure, milk and cheese contained gas-forming spores, demonstrating that the source of the contamination of the milk and cheese by spores was not from the DDGS but rather from the environment. Because all cheeses, including cheeses from cows not fed DDGS, exhibited body and texture defects, these findings do not implicate

DDGS, but rather spores not originating from DDGS, as the likely causative agent of the defects observed.

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Table 2.1. Summary of proximate analysis conducted on milk collected every week from individual cows (excluding first two week's data from each period) during DDGS feeding study

Component	0% DDGS	10% DDGS	20% DDGS	SEM	*P* -VALUE		
					Diet	Period	Diet* Period
pH	6.61[a]	6.63[a]	6.63[a]	0.01	0.269	0.028	0.465
FAT	3.45[a]	2.94[b]	2.67[b]	0.09	<0.0001	0.110	0.016
LACTOSE	5.07[b]	5.15[a]	5.17[a]	0.02	0.007	0.650	0.566
PROTEIN	3.58[b]	3.62[ab]	3.65[a]	0.02	0.041	0.002	0.574
SNF[1]	9.33[b]	9.47[ab]	9.55[a]	0.06	0.004	0.118	0.519

[a, b] -Concentrations not connected by the same superscripts are significantly different ($P < 0.05$).

Table 2.2. Summary of chemical analysis conducted on all baby Swiss cheeses made during DDGS study

	0% DDGS	10% DDGS	20% DDGS	SEM	*P* - VALUE	
Component					**Diet**	**Period**
pH	5.15	5.14	5.13	0.02	0.795	0.146
Moisture	41.28	41.67	41.36	0.39	0.803	0.956
Fat	30.18	29.70	29.50	1.39	0.945	0.780
Fat in solids	51.37	50.92	50.33	2.19	0.945	0.735
Protein	23.83	23.60	24.13	0.89	0.920	0.679
Ash	3.40	3.41	3.59	0.11	0.610	0.615
Calcium	7.40	7.49	7.84	0.23	0.597	0.880
Sodium	4.79	4.74	4.98	0.11	0.508	0.375
Salt	1.22	1.21	1.27	0.02	0.497	0.356
Salt in Moisture	2.94	2.90	3.07	0.03	0.208	0.173
Tbars[1]	0.10	0.10	0.08	0.007	<0.0001	<0.0001

[a] Levels not connected by same superscripts are significantly different.

[1]Thiobarbituric acid reactive substances

Table 2.3. Fatty acid composition of baby Swiss cheese from all three treatments

Fatty acid, wt %[1]	Treatments		
	Control	10%	20%
C4:0	2.3	2.0	2.0
C6:0	1.8	1.5	1.4
C8:0	1.1	0.8	0.8
C10:0	2.5	1.9	1.7
C12:0	2.8	2.3	2.1
C14:0	9.9	8.6	8.0
C15:0	0.9	0.8	0.7
C16:0	26.9	24.6	23.6
C17:0	0.5	0.4	0.4
C18:0	10.9	10.7	10.9
C19:0	0.2	0.1	0.0
C20:0	0.1	0.1	0.1
9c-C14:1	0.8	0.9	1.0
9c-C16:1	1.2	1.5	1.6
6t-C18:1	0.1	0.5	0.6
9t-C18:1	0.2	0.0	0.0
11t-C18:1	2.3	4.2	5.9
6c-C18:1	1.1	1.1	1.1
9c-C18:1	17.8	20.8	21.1
11c-C18:1	0.6	0.6	0.6
8c-C20:1	0.1	0.1	0.1
9c,12c-C18:2	2.3	2.6	3.0
9c,12c,15c-C18:3	0.3	0.2	0.2
8c,11c,14c-C20:3	0.1	0.0	0.0
5c,8c,11c,14c-C20:4	0.1	0.1	0.0

[1]Expressed as number of carbons: number of double bonds

A.  B. 

C.  D. 

Figure 2.1. Experimental baby Swiss cheese made during the study.

CHAPTER 3. NO ANTIMICROBIAL EFFECTS FROM ONE SOURCE OF COMMERCIAL DRIED DISTILLERS' GRAINS WITH SOLUBLES

A short communication to be submitted to *The Journal of Dairy Science*

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Abstract

Production of ethanol from corn involves fermentation by yeast. To minimize bacterial contamination, antibiotics are used in industrial ethanol fermentations, as unwanted bacteria compete with yeast for glucose and other nutrients during the yeast fermentation. Dried distillers' grains with solubles (DDGS) are one by-product of the ethanol production process that often is an economical protein source to include in animal feed. Because of fear of development of antibiotic resistant strains of bacteria from the use of antibiotics as a growth and/or performance enhancer in ruminant animals, the FDA has recommended that antibiotics be used judiciously. Residual antibiotics in DDGS could lead to inadvertent feeding of antibiotics to animals and is thus of concern. The objective of our study was to determine if a commercial DDGS used for a feeding study had any antimicrobial effect against select pathogens and milk spoilage organisms. Samples of the DDGS used in the feeding study and milk samples were sent to Iowa State University Veterinary Diagnostic Laboratory for drug screening. All the analytes tested, including virginiamycin, a commonly used antibiotic during ethanol fermentation process, were below the detection limits. Additionally, the impact of DDGS on the growth of isolates of *Salmonella* Typhimurium,

Listeria innocua, *E. coli ATCC 25922*, *Staphylococcus aureus*, *Pediococcus acidilacti*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bacillus licheniformis*, *Paenebacillus odorifier*, *Pseudomonas fluorescens*, and *Paenebacillus amyloliticus* were investigated using the disk diffusion seeded agar overlay method. DDGS (25 g) was mixed with water (225 ml) or 0.1% buffered peptone water (225 ml) using a stomacher to obtain the soluble fraction of the DDGS. Sterile paper disks were saturated with the resulting DDGS soluble fraction and placed on the lawn of overnight cultures (10^8 CFU/ml). Neither the buffered nor non-buffered DDGS solutions yielded any zone around any disk against any microorganism tested, indicating that the DDGS soluble fraction had no antimicrobial properties against any of the organisms tested. Additionally, because the buffered solution had no effect on the growth of microorganisms, the low pH of the soluble fraction of DDGS was not effective in inhibiting bacterial growth. The Iowa State University Veterinary Diagnostic Laboratory confirmed the absence of antibiotic residues in DDGS as well as in milk samples. These results indicate that the DDGS analyzed in this study can be used as livestock feed without fear of inadvertent feeding of antibiotics.

Key words: antimicrobial effect, distillers' grains, and antibiotic residues.

Introduction

Ethanol production from corn involves fermentation by yeast (*Saccharomyces cerevisiae;* U. S. Grains Council, 2010). After fermentation and distillation of ethanol, whole stillage (water and solids) is produced as a co-product. Through centrifugation, whole stillage is separated into thin stillage and coarse solids or wet distillers' grains (**WDG**, ~35% DM). These WDG are further dried in a rotary drier to form dried distillers' grains (**DDG**). The mixture of syrup (30% DM), obtained after evaporation of thin stillage, and DDG results

in the production of dried distillers' grains with solubles (**DDGS**, ~90% DM). As an often-economical energy, fiber, and protein source, DDGS have been used as feed for lactating dairy herds (Schingoethe, 2007, Giuntoli et al., 2009, Hoffman and Baker, 2011, Testroet et al. 2015; in press).

Virginamycin, a streptogramin antibiotic is used in industrial ethanol fermentations to control the growth of contaminating lactic acid bacteria that could interfere with desirable yeast fermentation (Hynes et al., 1997). Currently, the only antibiotic that has been provided a "no objection letter" from the FDA (Nov 16, 1993) for use in the ethanol fermentation process is virginiamycin. Virginiamycin is effective against seven strains of Lactobacilli during alcoholic fermentation, but is not effective against *L. rhamnosus*, *L. paracasei* and *L. plantarum* (Hynes et al., 1997). Virginiamycin has also been used in the livestock industry, particularly in ruminants, as a growth inhibitor of gram-positive bacteria. Virginiamycin has been shown to improve gain to feed ratios in beef cattle (Salinas-Chavira et. al., 2009) and to improve milk yield in lactating dairy cattle (Clayton et. al., 1999). Current FDA recommendations discourage the use of antibiotics for improving production parameters in livestock, and, instead, the FDA recommends "judicious" use of antibiotics in livestock to help prevent the development of antibiotic resistant microbes (FDA, 2014).

Because antibiotics are routinely used in the production of ethanol and, consequently, the production of DDGS, feeding DDGS could potentially result in inadvertent feeding of antibiotics to ruminant animals. A study conducted by FDA in 2012 (Fairfield, 2012) reported that 3 out of 28 (10.7%) samples DG had detectable antibiotic residues. Out of those 3 samples containing antibiotic residues, one sample contained 0.16-mg/kg virginiamycin M1 residue; a second sample contained 0.15 mg/kg of erythromycin; a third

sample contained approximately 0.15 mg/kg of virginiamycin M1 residue and 0.24 mg/kg of penicillin G. Determination of the presence or lack of antibiotics in distillers' grains is important, but it is additionally important to determine the biological activity of these antibiotics. In a more recent study by Paulus Compart et al. (2013), antibiotic residue concentrations in DG (wet: 79 samples, dry: 80 samples) were found to be very low and less than the maximum concentrations approved by FDA in feed for food-producing animals. Any residual antibiotics remaining in DDGS, however, could be detrimental in animal feeding programs because misuse or over-dosing of antibiotics could leads to development of antibiotic resistance in bacteria. Consumption of DDGS that contains antibiotic residues by food-producing animals could lead to development of resistance to those antibiotics (U. S. Grains Council, 2010). Inadvertent feeding of antibiotics to dairy cows through DDGS could have important implications for the dairy industry at large, particularly if any antibiotics in DDGS are transferred into milk. The objective of this study was to determine if one source of commercially available DDGS contained antibiotic residues and if these DDGS possessed any antimicrobial properties.

DDGS used in this study were acquired from Heartland Cooperative (Prairie City, IA). Dietary treatments were formulated for a dairy cow feeding study reported separately (Manimanna Sankarlal et al., 2015; in preparation, J. of Dairy Sci., Testroet et al., 2015; submitted to J. of Dairy Sci.). Diets included (1) total mixed ration (TMR) with no DDGS, (2) TMR with 10% DDGS by dietary DM, and (3) TMR with 20% DDGS. The TMR and DDGS composition were reported separately (Testroet et al., 2015; submitted to J. of Dairy Sci). Representative samples of feed from all the three treatments were collected, as

described in (Testroet et al., 2015; submitted to J. of Dairy Sci) and stored in Ziploc bags (S. C. Johnson & Son, Inc., Racine, WI) at -20°C until further analysis.

Samples of feed from all three treatment diets (TMR, 10% DDGS, 20% DDGS) were collected for microbial analysis. Three bags of feed (one bag for each treatment) were sent to the University of Nebraska-Lincoln, Food Processing Center for analysis. Aerobic mesophilic spore counts were determined on the feed samples based on AOAC 990.12. For the analysis, the feed samples were diluted (1:10 w:w) in Butterfield's phosphate buffer. The diluted feed sample (100 ml) was heated to 80°C and held at that temperature for 12 min. Serial dilutions of these samples were plated on Standard Methods Agar (SMA). The plates were incubated at 32°C for 48 hr (Wehr and Frank, 2004). After 48 hr, the plates were checked for growth and visually distinct bacterial isolates were streaked for purity on SMA.

At Iowa State University, samples of DDGS were diluted (1:10 w:w) with 0.1% Buffered Peptone Water (Fischer Scientific, Pittsburgh, PA) and mixed using a stomacher 400C (Seward®, Daive FL) for 30 sec. Twenty ml of the DDGS solution was heat treated at 80°C for 10min. The heat-shocked sample was plated on Tryptic Soy Agar (BD chemicals, Sparks, MD). The plates were stored at 35°C for up to 48 hr prior to evaluation for growth (Wehr and Frank, 2004).

Two representative samples of dried distillers' grains with solubles (DDGS) and six samples of milk (two samples from each of the three treatments) collected from cows fed with DDGS treatments were sent to the Cyclone Custom Analyte Detection Service (CyCads) at the Iowa State University Veterinary Diagnostic Laboratory (Ames, IA) to conduct full drug screening tests. Screening included quantification of Beta-lactams and Cephalosporins, Tetracyclines, Sulfonamides, Phenicols, Macrolides / Lincosamides /

Streptogramins, Quinolones, Aminoglycosides, and Anthelmintic (Antiparasitic) drugs using liquid chromatography and mass spectrometry.

The antimicrobial properties of the soluble fraction of DDGS was tested by determining the zone of inhibition by using the disk diffusion agar overlay method as described in Xia et al. (2012). For this method, overnight pure cultures (10^8 CFU/ml) were used to create lawns on solid agar. Pour agar plates were made by pouring (~15ml) using regular (1.5% agar; Fischer Scientific, Fair Lawn, NJ) and allowed to cool and harden. Specifically, Tryptic Soy Broth (BD chemicals, Sparks, MD) was used for *Salmonella* Typhimurium ATCC 14028, *Listeria innocua (*ATCC 30090, Microbiologics*)*, *E. coli ATCC 25922, Staphylococcus aureus* (ATCC 25923, Microbiologics); MRS Lactobacillus Broth (BD chemicals, Sparks, MD) was used for Pediococcus *acidilacti (NRRL)*, *Lactobacillus casei (Microbiologics)*, *Lactobacillus acidophilus (NRRL*); and Brain Heart Infusion Broth (BHI Teknova, Hollister, CA) was used for *Bacillus licheniformis* (FSL J3 – 0143; Cornell University, Ithaca, NY), *Paenebacillus odorifier* (FSL H8 – 0237; Cornell University, Ithaca, NY), *Pseudomonas fluorescens* FSL W5 – 0203; Cornell University, Ithaca, NY), and *Paenebacillus amyloliticus(* FSL H7 – 0689; Cornell University, Ithaca, NY). A 0.5 ml overnight culture suspension of each culture was added to 4.5 ml of tempered (50°C) 0.75% overlay agar of the same type and vortexed thoroughly before adding in each respective plate. This suspension mixture was then poured on the solid agar plates as an overlay and allowed to form a lawn of cells.

Dried distillers' grains and solubles (25 g) was mixed with 225 ml of water or 0.1% buffered peptone water (BPW; 225 ml; Fischer Scientific, Pittsburgh, PA) and stomached (400C Seward®, Daive, FL) for 30 sec. DDGS was mixed with 0.1% BPW in order to

neutralize the pH of DDGS (~3.5) and eliminate its pH effects on the microbes. Blank sterile paper disks (BD disgnostics, Sparks, MD) were saturated with the DDGS supernatant by adding as much as could be absorbed by the disk (around 20 microliter). Using sterile forceps, the disks were placed on the seeded agar overlay. After the disks attached to the surface of agar, the plates were inverted and incubated overnight at 35^0C. The zone of inhibition experiment was conducted in duplicate. Tetracycline disks (BD diagnostics, Sparks MD) were used as positive control and water-saturated disks were used as negative controls.

Feeding trials with the DDGS evaluated in the present study were conducted at the Iowa State University dairy farm for the purpose of evaluating the effect of DDGS on the quality of milk and baby Swiss cheese (Manimanna Sankarlal et al., 2015; in preparation, J. of Dairy Sci.). Anecdotal evidence during the feeding trials led us to believe that DDGS were protective against mastitis. On a full-herd basis, since the beginning of the trial, 482 unique Iowa State University dairy cows were milked. Of those, 60 (12.4%) were treated for mastitis, including the cows on our research. Out of the 30 cows on our 3-month trial, five contracted mastitis (17%). No mastitis occurred in the DDGS-fed cows. The main organisms involved in causing mastitis in these cows were Streptococcus spp., E. coli spp., and Klebsiella spp.

The bacterial counts in DDGS were too low to count (<5 CFU/ml, data not shown). The high temperatures (up to 426.6°C) associated with the DDGS drying process likely only partially explain these results (Pfizer Animal Health, Lactrol Technical Information Pamphlet). Although Pedersen et al. (2004) did not find microorganisms in the wheat WDG samples collected directly out of the distillation column, storage in unclean environments leads to increases in microbial loads. Up to 8.4 CFU/ml of Lactobacilli (including species

like *Lactobacillus amyloliticus, Lactobacillus panis, Lactobacilli pontis*) was found in wheat WDG when stored over time (more than 5 weeks, Pedersen et al., 2004). Microorganisms naturally present in the environment could contaminate the DG, and if antibiotics are also present in DDGS, such compounds could lead to antibiotic resistant strains of bacteria resulting from incidental contact during storage. Jacob et al., (2008) hypothesized that administering feed containing antibiotics to cattle would lead to development of antibiotic resistant bacteria in the gut of cattle; they, however, found no direct correlation between feeding DG containing antibiotics and development of antibiotic resistance in the gut of cattle. Based on their risk assessment review, Paulus Compart et al. (2013) concluded that the health risk to humans of antibiotic resistance from feeding DG to cattle is very minimal compared with other external factors like prevalence of bacteria in feed that acquired antibiotic resistance genes from the environment. Because over-use and misuse of antibiotics can lead to antibiotic resistance and so-called "super-bugs", even minimal amounts of antibiotics in DDGS would be undesirable and potentially have negative effects.

Indeed, once mixed with TMR, which is not stored in a sterile environment, several species of bacteria were isolated from the TMR, 10% DDGS diet and 20% DDGS diet. Bacterial isolates were not identified at the molecular level but were determined to be, primarily, *Bacillus licheniformis* and *Bacillus brevibacillus*, along with fewer amounts of *Paenibacillus* spp., *Bacillus pumilus* and *Bacillus subtilis*. Total aerobic count in the TMR was 6.69 $\log_{10}$ CFU/g. The feed containing 10% DDGS (5.98 $\log_{10}$ CFU/g) and 20% DDGS (5.81 $\log_{10}$ CFU/g) substitution in TMR showed about one log reduction in microbial counts when compared to the control feed (TMR), which is more than would be expected by dilution

(10% and 20% substitution of TMR) effect alone. The decrease in the counts was hypothesized to be because of an antimicrobial effect of DDGS substitution in the TMR.

Although utilization of antibiotics in ethanol production is strictly controlled by FDA, there are still concerns about antibiotic residues remaining in distillers' co-products (Paulus Compart et al., 2013). However, in our study the concentration of all tested analytes in DDGS (Table 3.1) and in the milk (Table 3.2) was below the detection limit Concentration of virginiamycin, the antibiotic commonly used in the ethanol production process, was less than 1 mg/kg. The FDA's Center for Veterinary Medicine recommended the maximal concentration of residual virginiamycin in distillers' by-products be 0.2 to 0.5 mg/kg and about 2 to 6 mg/kg of virginiamycin should be allowed during the fermentation process (National Grain and Feed Association, 2009). The FDA-approved maximal concentration of virginiamycin for use in finishing cattles' feed is 22.5 g/ton. In 2012, research conducted by the FDA found virginiamycin residues in two DG samples out of 28 distillers' grains samples (7.1%) at a concentration approximately 0.16 mg/kg and 0.15 mg/kg. The second sample contained penicillin G (0.24 mg/kg) along with virginiamycin. A more recent study conducted by Paulus Compart et al. (2013) found virginiamycin residues in two feed samples of dried distillers' grains (DDG) (0.6 mg/kg and 0.5 mg/kg). Antibiotic residues in the previous and present work were below the current FDA approved maximal levels. It should be noted that even at low concentrations, toxic effects might result from the combined action of antibiotics. For instance, macrolide antibiotics such as erythromycin and virginiamycin, have the chance of inducing monensin toxicity by delaying its clearance in the liver when supplemented (Basaraba et al., 1999). Basaraba et al. (1999) reported a case where monensin

caused death of cattle fed DDG containing macrolide antibiotic (50 to 1500 mg/kg) combination of erythromycin, clarithromycin, and others).

The supernatant of DDGS used in the study did not form any zones of inhibition; no antimicrobial effect was found against *Listeria innocua*, *Salmonella* Typhimurium, *E. coli ATCC 25922, Staphylococcus aureus*, *Pediococcus acidilacti, Lactobacillus casei*, *Lactobacillus acidophilus*, *Bacillus licheniformis*, *Paenebacillus odorifier*, *Pseudomonas fluorescens*, and *Paenebacillus amyloliticus*. *Bacillus licheniformis* and *Paenibacillus* were the only organisms identified in the TMR, which were also evaluated in the zone of inhibition study. Thus, the antimicrobial activity of DDGS against other organisms found in TMR would need to be confirmed to ensure that DDGS did not have any antibiotic effect against any of the bacteria in the TMR. Similarly zone of inhibition of DDGS against organism that caused mastitis in the Iowa State University dairy farm cows needs to be performed to ensure that DDGS was not protective against mastitis. Our results were indeed very similar to Paulus Compart et al. (2013) who reported that out of 159 samples of DG (wet: 79 samples, dried: 80 samples) tested in the study, only one DG sample inhibited the growth of *E. coli* ATCC 8739. However, this DG sample did not contain any detectable antibiotic residues. The source of antimicrobial effect against *E. coli* ATCC 8739 was unclear.

A commercial DDGS used for dairy cattle feeding research at Iowa State University did not possess a detectable level of antibiotics or antimicrobial compounds that were active against *Salmonella* Typhimurium, *Listeria innocua*, *E. coli ATCC 25922*, *Staphylococcus aureus*, *Pediococc*us *acidilacti*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bacillus licheniformis*, *Paenebacillus odorifier*, *Pseudomonas fluorescens*, and *Paenebacillus*

amyloliticus. The DDGS used in the study will not likely contribute negatively to the health of the cows or to antibiotic resistance in the herd.

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Table 3.1. Concentration of analytes in DDGS samples

		Concentration in DDGS, ppm	
Analytes	Limit of Detection, ppm	DDGS 1	DDGS 2
Bacitracin Methylene Disalicylate	10	<10	<10
Bacitracin Zinc	10	<10	<10
Bambermycins	2	DNT[1]	DNT[1]
Carbadox	0.125	<0.125	<0.125
Chlortetracycline	2	<2	<2
Florfenicol	0.5	<0.5	<0.5
Lincomycin	8	<8	<8
Naracin	1	DNT[1]	DNT[1]
Oxytetracycline	2	<2	<2
Penicillin	2	<2	<2
Sulfamethazine	1	<1	<1
Sulfathiazole	1	<1	<1
Tiamulin	0.125	<0.125	<0.125
Tilmicosin	1	<1	<1
Tylosin	0.5	<0.5	<0.5
Tylvalosin	0.5	DNT[1]	DNT[1]
Virginiamycin	10	<1	<1

[1]DNT – did not determine

Table 3.2. Concentration of analytes in milk samples

Antibiotic/Antiparasitic assay	Assay LOQ[1] (ppb)	Safe/Tolerance level in milk* (ppb)	Assay result** (ppb)
Beta-Lactams and Cephalosporins			
Amoxicillin	0.5	10	ND[2]
Ampicillin	0.5	10	ND
Ceftiofur	0.5	100	ND
Desfuroylceftiofur (DFC)	0.5	100	ND
DCCD (Ceftiofur metabolite)	0.5	TNE[3]	ND
Cephapirin	0.5	20	ND
Cloxacillin	0.5	10	ND
Penicillin G	0.25	5***	ND
Tetracyclines			
Chlortetracycline	2.5	300	ND
Oxytetracycline	2.5	300	ND
Tetracycline	2.5	300	ND
Doxycycline	2.5	TNE	ND
Sulfonamides			
Sulfachlorpyridazine	0.5	10***	ND
Sulfadiazine	0.5	10***	ND
Sulfamerazine	0.5	10***	ND
Sulfadimethoxine	0.5	10***	ND
Sulfapyridine	0.5	10***	ND
Sulfamethazine	0.5	10***	ND
Sulfaquinoxaline	0.5	10***	ND

Table 3.2 . Concentration of analytes in milk samples (continued)

Sulfathiazole	0.5	10***	ND
Macrolides, Lincosamides and Streptogramins			
Erythromycin	2.5	50***	ND
Tulathromycin	2.5	TNE	ND
Tilmicosin	2.5	TNE	ND
Tylosin	2.5	50	ND
Lincomycin	0.5	TNE	ND
Pirlimycin	0.5	40	ND
Virginiamycin	2.5	TNE	ND
Quinolones			
Ciprofloxacin	0.5	TNE	ND
Enrofloxacin	0.5	TNE	ND
Norfloxacin	0.5	TNE	ND
Sarafloxacin	0.5	TNE	ND
Phenicols			
Chloramphenicol	NA[4]	TNE	ND
Florfenicol	0.5	TNE	ND
NSAIDs & Miscellaneous			
Flunixin	0.1	2	ND
5-Hydroxy Flunixin	0.1	2	ND
Meloxicam	0.1	TNE	ND
Bacitracin	2.5	500	ND
Tiamulin	0.1	TNE	ND
Ractopamine	0.1	TNE	ND
Tripelenamine	0.1	20	ND

Table 3.2. Concentration of analytes in milk samples (continued)

Antihelmintic (Antiparasitic)			
Doramectin	0.5	TNE	ND
Moxidectin	0.5	TNE	ND
Ivermectin	0.5	TNE	ND
Thiabenzadole	2.5	50	ND

[1] LOQ= Limit of Quantification

[2]ND= Not Detected

[3]TNE= Tolerance Not Established

[4]NA= Not Available

* Tolerance based on CFR 21 Part 556 'Tolerances for residues of New Animal Drugs in Food'. Tolerances are only applicable to milk

** One column indicates the assay result of all the six milk samples analyzed (2 samples from each of the three treatments (0% DDGS, 10% DDGS, 20% DDGS))

*** Amounts are "safe levels" not tolerances

CHAPTER 4. GENERAL CONCLUSION

Feeding 20% DDGS as part of a TMR decreased percent fat and increased percent lactose and percent protein in milk. Cheese fatty acid profile revealed that long chain unsaturated fatty acids increased numerically with DDGS feeding while short chain and medium-chain fatty acids decreased. The DDGS source used for this research did not contain gas-forming spores, yet TMR, manure, milk and cheese contained gas-forming spores, demonstrating that the source of the contamination of the milk and cheese by spores was not from the DDGS but rather from the environment. Because all cheeses, including cheeses from cows not fed DDGS, exhibited body and texture defects, these findings do not implicate DDGS, but rather spores not originating from DDGS, as one likely causative agent of the defects observed. Finding the solution to eliminate this defect in cheese could be very difficult because various factors including changes in milk composition and their combined effects could contribute to this problem at various stages of cheese making.

The commercial DDGS used for this dairy cattle feeding research at Iowa State University did not possess a detectable level of antibiotics or antimicrobial compounds that were active against *Salmonella* Typhimurium, *Listeria innocua*, *E. coli* specify *ATCC 25922*, *Staphylococcus aureus*, *Pediococc*us *acidilacti*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bacillus licheniformis*, *Paenebacillus odorifier*, *Pseudomonas fluorescens*, and *Paenebacillus amyloliticus*. The DDGS used in the study will not likely contribute negatively to the health of the cows or to antibiotic resistance in the herd.

CHAPTER 5. FUTURE RESEARCH

Feeding 20% DDGS led to a significant increase in lactose content in the milk. If lactose is not adequately catabolized at the end of dipping process during cheese making, high residual lactose in the milk may enable lactic acid bacteria to participate in secondary fermentations and affect the eye formation. To test this hypothesis, future research is designed to vary the amount of lactose in milk and make baby Swiss cheese from it to evaluate the impact of lactose on eye formation.

On the same note, presence of residual lactose in milk may act as a substrate for non-starter fermentation by spores such as *Clostridium tyrobutyricum*. To investigate this hypothesis, spores can be spiked in the milk along with varying lactose content and cheese can be made from this milk. The effect of increased lactose content in the presence and absence of spores on the composition of baby Swiss cheese can be studied for in-depth understanding of the behavior of spores towards varying lactose content and secondary fermentation.

The unsophisticated hydraulic press used in the current study led to the question about whether it was a contributor to undesirable eye formed in the cheeses. To eliminate this, appropriately designated cheese presses will be used to make future cheeses.

Presence of higher unsaturated fatty acids in the milk and eventually in cheese significantly lowers the firmness of cheese producing a softer cheese texture (Jaros et al., 2001). Softer cheese body could lead to less elastic structure that is unsuitable to accommodating eyes leading to poor eye development. In future, cow diets can be supplemented in order to decrease unsaturated fatty acids in milk and analyze the change in firmness of cheese.

A formal trained sensory evaluation panel can be conducted to compare the quality of baby Swiss cheese from different treatments. Aroma (propionic acid, lactic acid, hydrogen sulfide, rancid, nutty, sweet, cooked), texture (hardness, moist, dry, elastic, rubbery), characteristic of eyes (size, number, shape) can be assessed using a trained sensory panel. Additionally the texture of cheese can also be studied using texture analyzer for comparison.

Through our short communication, we were able to analyze the effect of DDGS soluble fraction (diluted in water, BPW) against selected organisms. In order to understand the complete mechanism or in order to eliminate the suspicion about antimicrobial effects of DDGS against target organisms, the DDGS could be subjected to more rigorous extraction steps using alcoholic solvents such as ethanol, hexane etc. according to the type of extract of interest. The antimicrobial effect of DDGS extract can be tested against other cultures that are commonly found in milk, meat, and gut of food producing animals using virginiamycin as a positive control.

APPENDIX SWISS CHEESE MAKE SHEET

Date: _____ vat #: _____Cow group (DDGS diet): _____ % fat: _____ % protein: _____ ratio: _____

Total milk (after past.): _____ Inch marker (amt. of milk): _____ minus 25%: _____ plus 5%: _____plus 10%: _____

S. theromophilus: 0.8 g/100#: amount: _____ g L. *helveticus*: 0.08g/100#: amount: _____ g

Propionibacteria: 0.5 g/100#: amount: _____ g Coagulant: 4 ml/100#: amount: _____ mL 1 : 40: _____ water

Brine: (4.6 kg/20 L NaCl, (23%) + 76 mL $CaCl_2$) Date of fresh brine: _____

Target time	Actual reached	Estimated time	Actual time	Process Step	Temp.	pH
-4.00		8 AM		Filter, separate skim/cream; standardize to 0.88 fat/protein	X	
-2:00		10		Pasteurize 164°F/73.5°C, 15 sec (flow rate 4.0); exit 98°F/37°C		X
-1:00 0:00		10:00 11 PM		Fill vat and cool to 91°F/33°C. Add cultures; ripen for 45 min.		
0:45		11:45		Add rennet and stir <2 min; allow 25-30 min set	X	X
1:10		12:10		Check curd (set medium firm)	X	X
1:15		12:15		Cut 1 (horizontal): Slow speed until coagulum moves	X	X
1:17		12:17		Cut 2 (vertical): Curd should be ¼ to 3/8 in size. Heal 2 min.	X	X
1:24		12:24 1:00		Start fore work: (no heat; stir before cooking). End fore work: 35:00 min	———	———
2:00		1:00		Pre-draw 1: Remove 25% of vat volume in whey.		X
2:05		1:05		Stir After Pre-draw: Add 3-5% water @ 91°F/33°C		X
2:10		1:10		Cook 1: 5:00 min or until temp is 93°F/34°C		X
2:15		1:15		Cook 2: 5:00 min or until temp is 98°F/37°C		X
2:20		1:20		Cook 3: 5:00 min or until temp is 103°F/40°C		X
2:25		1:25		Cook 4: Increase temp more rapidly to 115°F/46°C over 10 min period. At 110°F/44°C, slowly add 10% of the original vat volume in water at 115°F/46°C to facilitate temperature rise.	———	X
2:35		1:35		Cook 5: Decrease cook speed to hit temp target of 118°F/47.8°C. If temp exceeds 119°F, add cold water.		X
2:40 3:20		1:40 2:20		Post work: stir after cook: 42:00 min (Start 42 min when 47°C is reached (ok for slight drop or rise)).	———	X
3:20		2:20		Pre-draw 2: Remove enough whey to cover curds (~3 inches). Whey pH Should be 6.4 before next step.		
3:25		2:25		Stir while draining curds and whey into clear plastic molds placed in clear basin. Keep curds	X	X

				under whey.		
3:30		2:30		Press curds (under whey) with basins of water for 15 min.	X	
3:45 5:45		2:45 4:45		Drain whey-filled basin completely. Press inside clear basin for 5 hr. Check/flip/check pH and discard whey after 2 hr.	X	
8:45 10:45		7:45 9:45		Fermentation: Remove pressure and allow fermentation to complete in clear basin (~10 hr @ ~82°F/28°C). Flip/drain whey after 2 hr.	X	
~18:00		~6 AM		Cheese pH should be 5.25 pH prior to brine. Brining: 12 hr @ 50°F/10°C (assuming ~10lb block).	X	
~30:00		~6 PM		Package in vacuum sealed barrier bags.	X	X
Day 8				Precool: @ 50°F/10°C for 7 days.		X
Day 29				Warm room: 21 days @ 72°F/22°C. Flip cheese weekly.		X
Day 60				Cool to 35°F/4°C when eyes reach ½-5/8 inch (60 days)		X

Total time fermented until pH 5.25 reached: _____

Total weight of cheese (and extra curds): empty container(s): total weight cheese:

yield (%):