THE GRAVITY SEPARATION OF FAT, SOMATIC CELLS, BACTERIA, AND SPORES IN MILK

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ABSTRACT

Gravity separation has been used for traditional cheese making of Grana Padano and Parmigiano-Reggiano cheeses. This process allows the whole milk to be standardized to a 2.2% fat. Traditional cheese makers have stated that they continue to use gravity separation as opposed to more modern centrifugal separation due to the difference in quality and flavor of cheese.

Our first objective was to determine if immunoglobulins (Ig) play a role in the gravity separation (rising to the top) of somatic cells (SC) in skim milk. Other researchers have shown that gravity separation of milk fat globules is enhanced by IgM. Our recent research found that bacteria and SC gravity separate in both raw whole and skim milk and that heating milk to >76.9^{o}C for 25s stopped gravity separation of milk fat, SC, and bacteria. Bovine colostrum is a good natural source of Ig. An experiment was designed where skim milk was heated at high temperatures (76°C for 7 min) to stop the gravity separation of SC and then colostrum was added back to try to restore the gravity separation of SC in increasing increments to achieve 0, 0.4, 0.8, 2.0, and 4.0 g/L of added Ig. The milk was allowed to gravity separate for 22 h at 4°C. The heat treatment (76°C for 7 min) of skim milk was sufficient to stop the gravity separation of SC. The treatment of 4.0 g/L of added Ig was successful in restoring the gravity separation of SC as compared to raw skim. Preliminary spore data on the third replicate suggested that bacterial spores gravity separate the same way as the SC in raw, and heated skim with 4.0 g/L of added Ig. There is strong evidence that Ig are at least one of the factors necessary for the gravity separation of SC and bacterial spores.

Our second objective was to determine the role that Ig and somatic cells (SC) play in the gravity separation of milk. There were 9 treatments: (1) low temperature pasteurized (**LTP**) (72°C for 17.31s) whole milk, (2) LTP (72°C for 17.31s) whole milk with added bacteria and spores, (3) recombined LTP (72°C for 17.31s) whole milk with added bacteria and spores, (4) high temperature pasteurized (**HTP**) (76°C for 7 min) whole milk with added bacteria and spores, (5) HTP (76°C for 7 min) whole milk with added bacteria and spores and added colostrum, (6) HTP (76°C for 7 min) centrifugal separated gravity separated (**CS GS**) skim milk

with HTP (76°C for 7 min) low SC cream with added bacteria and spores, (7) HTP (76°C for 7 min) CS GS skim milk with HTP (76°C for 7 min) high SC cream with added bacteria and spores, (8) HTP (76°C for 7 min) CS GS skim milk with HTP (76°C for 7 min) low SC cream with added bacteria and spores and added colostrum, and (9) HTP (76°C for 7 min) CS GS skim milk with HTP (76°C for 7 min) high SC cream with added bacteria and spores and added colostrum. The milks in 9 treatments were gravity separated at 4°C for 23 h in glass columns. The presence of both SC and Ig were necessary for normal gravity separation (i.e., rising to the top) of fat, bacteria, and spores in whole milk. The presence of Ig alone without SC was not sufficient to cause bacteria, fat and spores to rise to the top without SC. The SC may provide the buoyancy required for the aggregates to rise to the top due to gas within the SC. More research is needed to understand the mechanism of the gravity separation process.

BIOGRAPHICAL SKETCH

Stephanie Rose Geer was born in July of 1989 in Johnson City, NY. After graduating in 2007 co-salutatorian of her high school class, Seton Catholic Central High School, she went on to attend the The College at Brockport, SUNY. While at the College at Brockport, she enrolled as a chemistry major with an economics minor. During the summer of 2008 and the summer of 2009, she performed research in an electrochemistry graduate lab at Binghamton University. She discovered her passion for food science after participating in the Cornell Food Science Summer Scholar Program in the summer of 2010.

In addition, she was a four-year member of the varsity softball team, playing the position of pitcher, and was team captain her senior year. She received several athletic awards, including the College at Brockport Female Scholar Athlete of the year, given to one female athlete out of the whole athletic department, and the CoSida Academic All-American 1st Team, chosen out of all female softball athletes in Division 1, II, and III, becoming only the fourth student athlete at the College at Brockport to receive that award. She finished her career second for all-time strickout list as a pitcher.

She graduated *summa cum laude* in 2011 with a B.S. degree in chemistry, with ACS certification. She received several prominent awards including the SUNY Chancellor's Award of Excellence given to only four students at the College at Brockport, the Chemistry Departmental scholar award for having the highest GPA out of the graduating chemistry majors, and the School of Science and Mathematics Award, given to one graduating senior from the School of Science and Mathematics.

She enrolled at Cornell University in the M.S. program in food science in the fall of 2011. She received the WNY IFT Outstanding Achievement in Food Science and was inducted into the Phi Tau Sigma Food Science National Honor Society. She also participated in two product development teams while at Cornell. When she graduates, she hopes to get a job in industry where she can combine her knowledge of chemistry with her passion of food science. In the summer of 2014, she'll be marrying her fiancée, who she met while an undergraduate.

I dedicate this work to my parents, Les and Sue Geer, and my fiancée of five years, Mike Gonzalez, for their unwavering support in helping me reach my goals.

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LIST OF ABBREVIATIONS

ANOVA……... Analysis of variance

BHI……... Blood heart infusion

CS……Centrifugal separated

GS……..Gravity separated

HTP…….High temperature pasteurized

Ig…….Immunoglobulin

LTP……..Low temperature pasteurized

MFGM……...Milk fat globule membrane

MSC……..Mesophilic spore count

P……...Level of significance

SAS……..Statistical analysis system

SC……...Somatic cell

SCC……..Somatic cell count

SMB…….Skim milk broth

SPC……Standard plate count

CHAPTER ONE

Background on the Gravity Separation of Fat, Somatic Cells, Bacteria and Spores in Bovine Milk

HISTORY OF GRAVITY SEPARATION

Observations on the Gravity Separation of Milk Fat

A well-known phenomenon in bovine milk is the gravity separation, or rising to the top, of fat globules when whole milk is allowed to stand unagitated. A cream layer is visible at the top of the milk due to the clustering and rising of fat globules (Sharp and Krukovsky, 1939). Not all animal milks will gravity separate. For instance, camel, goat, and buffalo milks do not gravity separate as well as bovine milk (Fahmi, 1951; Jenness and Parkash, 1971; Farah and Rüegg, 1991). Farmers knew about this phenomenon for years and it was reported as early as 1859 that attempts were made to use centrifugal force to separate cream and milk. It was not until 1877 that a practical cream separator was developed (Frederiksen, 1885).

The creaming of fat was attributed to the difference in specific gravity between the fat globules and milk serum (Frederiksen, 1885). It was noted that the temperature of gravity separation affected the cream layer, and that the optimal temperature to produce cream for butter making was 12 to 16°C (Knisely, 1883). However, other researchers have stated that the milk should be cooled quickly to 4°C to control microbial growth during storage (Dahlberg and Marquardt, 1931). Masuda (1983) found as the temperature of gravity separation increased, the weight of the cream layer and the amount of fat (grams) in the cream layer decreased and the percent fat of the cream layer increased. During the gravity separation of whole milk, a cream layer would be visible as early as 2 to 4 hours at 4°C (Dahlberg and Marquardt, 1931). Heating milk between 50 – 60°C for 5 minutes restored the creaming ability of milks that did not cream well (Dahlberg and Marquardt, 1931; Mertens, 1933a). Similarly, research by Caplan et al. (2013) observed that there was more gravity separation of fat in whole milk that was pasteurized at 72.6°C for 25s than in raw whole.

Other observations showed that a solution of 0.1% sodium or potassium hydrate in milk would stop the gravity separation of fat, indicating the possibility that gravity separation of fat is pH dependent (Babcock, 1889). The container the milk was held in during gravity separation affected the rate the fat globules rise, since a shallower container had less distance that the fat needs to rise (Babcock, 1889). The larger fat globules rise to the top the quickest, so after gravity separation took place, the bottom skim portion contains fat globules with smaller particle diameters (Ma and Barbano, 2000). Some evidence showed the final volume of the cream layer could be predicted from the starting fat percentage of the milk (Dahlberg and Marquardt, 1931), although this might not be the case for milk from individual cows (Hammer, 1916). In addition, there are within and between breed variations for the rising of the fat (Dahlberg and Marquardt, 1931).

Gravity separation is an important concept to traditional cheese makers of Grana Padano and Parmigiano-Reggiano cheeses in Italy, who take advantage of the rising of the fat in raw whole milk to standardize their cheese (Gobbetti, 2004). However, more seems to be going on than just the fat rising to the top as traditional cheese makers have stated that they continue to use gravity separation as opposed to more modern centrifugal separation due to the difference in quality and flavor of their cheese (Caplan, et al., 2013). The safety record of these cheeses is also very high, even though they are made with raw milk (Johnson, et al., 1990). The gravity separation process needs to be better understood to explain the unique characteristics of the cheeses.

Several theories, including electrokinetic potential of fat globules, interfacial tension, stickiness and state of hydration of the adsorbed membrane, and a agglutination process were developed to try to explain why fat gravity separates since it was determined that the fat globules rose quicker than predicted by Stoke's Law (Troy and Sharp, 1928; Dunkley and Sommer, 1944). If the fat globules clustered together, the faster rising could be explained (Troy and Sharp, 1928). It was discovered that the fat globules in milk aggregate together in a similar fashion to aggregation of components in blood (Babcock 1889). When a small amount of blood serum was

mixed with fat globules, the fat globules would aggregate the same way they aggregate in skim milk (Babcock 1889).

Possible Role of IgM in the Gravity Separation of Fat

It was noted that colostrum contained higher amounts of Ig than milk, around 12 to 15% (Babcock, 1889). When colostrum was allowed to gravity separate, high concentrations of Ig slowed the gravity separation process and had a cream layer that was less concentrated, which may in part be due to an increase in viscosity from the higher protein concentration (Babcock, 1889). Moody et al. (1951) had similar observations of the gravity separation of fat in colostrum, and noted that when total protein in the colostrum exceeded 16% or Ig concentration exceeded 8%, no cream layer would appear. Mertens (1933b) found when colostrum or a 1% or 5% milk Ig solution was added to milk, it increased the speed of the fat rising to the top. Jenness and Parkash (1971) observed that colostrum could restore the gravity separation of fat in whole milk that had been pasteurized at 85°C for 30 min. This indicates that there is something in colostrum and blood serum that was necessary for gravity separation of fat to take place.

It was later thought that euglobulins played some role in the process, although it was not well understood (Payens et al., 1964). Masuda (1983) measured the total amount of Ig in the cream and skim layer after the gravity separation of milk, and as the temperature increased, the amount of Ig in the cream layer decreased while the amount in the skim layer increased (Masuda, 1983).

The agglutinating factor was determined to be in the skim milk portion as opposed to the cream portion when the warm milk was centrifugally separated (Farah and Rüegg, 1991; Jenness and Parkash, 1971; Dunkley and Sommer, 1944). When cold milk was centrifugally separated, the reverse was found (Dunkley and Sommer, 1944). Sharp and Krukovsky (1939) theorized that the agglutinin was absorbed onto solid fat globules but not onto liquid fat globules, which would explain the difference observed between cold and warm centrifugal separation. This agglutinating process in gravity separation was later termed cold agglutination (Huppertz and

Kelly, 2006). This process was found to be heat sensitive, as the creaming process begins to decrease at temperatures greater than 63°C when the milk is held for 30 mins (Rowland, 1937).

Orla-Jensen (1929) reported that it wasn't the MFGM that was changed during the heat process, but something in the milk plasma also found in bovine blood that can cause agglutination. The decrease of gravity separation of fat at higher heating temperatures prior to gravity separation was proportional to the denaturation of albumin and Ig (Rowland, 1937). At high temperatures, the effect of heat was much quicker, as only a few seconds at 75°C were need to stop gravity separation (Samuelsson et al., 1954), similar to research by Caplan et al. (2013) who found that 76.9°C for 25s stopped gravity separation of fat. These temperatures are above the legal HTST pasteurization temperatures of 72°C for 15s (Pasteurized Milk Ordinance, 2011) which explains why pasteurizing milk at this temperature will still have the gravity separation of fat.

The results indicate that at least one of the necessary components of gravity separation is heat labile. Ig are a good candidate as the heat-labile component of the gravity separation of fat based on the fact that they are found in blood serum, colostrum, and milk of bovines and they have the capability to form agglutinations (Hurley and Theil, 2011).

Further experimental research identified IgM as the likely Ig involved in the gravity separation of fat (Euber and Brunner, 1984). Only 7% of the total amount of IgM present in bovine milk is needed for gravity separation (Euber and Brunner, 1984). When IgM is added to milk that had high heat treatment, it restored the gravity separation of fat. When IgG was added to that same milk, no restoration of gravity separation was observed (Euber and Brunner, 1984). Similarly, when IgM was removed using an IgM specific antiserum, the gravity separation of fat did not occur. Yet when an IgG specific or IgA specific antiserum was added to milk, there was no change in the gravity separation of fat (Euber and Brunner, 1984). Honkanen-Buzalski and Sandholm (1981) theorized that IgA was responsible for the gravity separation of fat due to IgA's strong association with fat globules and cream. Most literature accepts IgM as the more

likely agglutination factor of fat (Huppertz and Kelly, 2006; Fox and McSweeney, 1998). Bovine Ig will be discussed more in depth later in this section.

Possible role of MFGM in the Gravity Separation of Fat

MFGM is the membrane that surrounds the fat globule. It is composed of proteins/enzyme, lipids, and carbohydrates (Keenan and Mather, 2006) and originates from the mammary secretory cell membrane. Homogenization stops the gravity separation of fat (Hammer, 1916). Homogenization changes the protein composition of the MFGM material, as less original protein is seen on the membrane after homogenization (Lee and Sherbon, 2002). The average size of the fat globules decreases and the surface area increases after homogenization (Lee and Sherbon, 2002). Dunkley and Sommer (1944) theorized that because homogenization caused an increase of surface area on the fat molecules, it would require greater amounts of agglutinin in order for gravity separation to take place. Research by Darling and Butcher (1978) showed that caseins preferentially adsorbed onto the new fat membrane after homogenization. The ratio of protein to fat in the homogenized milk fat globules is much higher, thus increasing the density of the small fat globules.

Euber and Brunner (1984) added IgM and skim milk membrane separately to homogenized milk. The addition of IgM had no effect on the gravity separation of fat in homogenized milk, but the addition of the skim milk membrane restored the gravity separation of homogenized milk. There are structural differences in the skim milk membrane and MFGM, with differences in amount of cholesterol, phospholipids, carbohydrate, lipid class distribution, enzyme activities, and protein composition (Kitchen, 1974). There are also similarities between the skim milk membrane and MFGM that suggests most of the skim milk membrane comes from the MFGM (Wooding, 1974). The differences could arise from the fragmentation of the MFGM in the skim phase (Wooding, 1974). IgM has been found to specifically interact with the MFGM by binding to carbohydrates on the membrane (Euber and Brunner, 1984).

Use of Gravity Separation in the Cheese Industry

The concept of fat gravity separating in milk has been used for traditional cheese making of Grana and Parmigiano-Reggiano cheeses (Gobbetti, 2004). Traditional Parmigiano-Reggiano cheeses are made by allowing raw milk to gravity separate overnight at 20°C, removing some of the cream layer, and mixing in fresh raw whole milk to obtain a 2.4-2.5% fat milk. The milk for traditional Grana Padano cheeses are prepared by gravity separating raw whole milk at 12-15°C for 12 hours and then removing the fat layer to get a 2.1-2.2% fat milk (Gobbetti, 2004). Traditional cheese makers have stated that they continue to use gravity separation as opposed to more modern centrifugal separation due to the difference in quality and flavor of the milk (Caplan et al., 2013). This might be due to the possible removal of spores during gravity separation.

GRAVITY SEPARATION OF BACTERIA, SPORES, AND SOMATIC CELLS

Gravity Separation of Bacteria and Spores

The unique flavor and aroma characteristics of traditional Grana Padano cheese (Moio and Addeo, 1998) may be also due to the fact that bacteria rise to the top during gravity separation along with the fat. Carminati et al. (2008) added 4 different pathogenic bacteria, *L. monocytogenes, Salmonella spp., Staph, aureus,* and *E. coli* O157:H7, to raw whole milk and observed that all 4 strains rose to the top after gravity separation at 8°C for 16 h. Franciosi et al. (2011) analyzed the psychotrophic microbial population before and after creaming of raw milk. They found that most of the total psychrotrophic bacteria were in the cream after gravity separation, but it also depended on the species of bacteria. *Flavobacteriaceae* and *Pseudomonadacea* were found more concentrated in the cream, *Streptococcaceae* and *Enterobacteriaceae* were found more concentrated in the skim, and *Moraxellaceae* was found in equal concentration in the skim and cream.

Other researchers have also reported bacteria rising to the top in bovine milk during gravity separation. Anderson (1909) recommended for infant formulas to not be made with cream from gravity separation due to the high bacteria count. He observed 10 to 500 times as

many bacteria were in the cream layer as compared to the starting milk after gravity separation. Dellaglio et al. (1969) found that after gravity separation, *Steptococcus cremoris*, *Staphylococcus aureus*, *Acinetobacter*, *Escherichia coli*, *Pseudomonas flourescensm*, and *Flavobacterium* were more concentrated in the cream. Abo-Elnaga et al. (1981) found similar bacterial species differences with gravity separation, with *Streptomyces* not gravity separating and *E. coli, Streptoccoccus lactis, Saccharomyces sp., Bacillus subtilis,* and *Micrococcus sp.* showing gravity separation, although the percent found in the cream layer was different between the species. It's been estimated that between 67 to 99% of the initial bacteria rises to the cream layer after gravity separation (Abo-Elnaga et al, 1981).

There is also competition between bacteria and fat, as milk that is higher in bacteria counts will have a higher percentage of total bacteria at the top but a lower percent of total fat at the top compared to a milk with a lower starting bacteria count (Caplan et al., 2013). Stadhouders and Hup (1970) showed that bacteria agglutinate in milk and become attached to the fat globules, suggesting a similar agglutinating process for fat and bacteria.

In addition, *Clostridium tyrobutyricum* spores were more concentration in the cream after gravity separation (Dellaglio et al., 1969). Rossie (1964) found that some spore forming clostridia bacteria rose to the top during gravity separation. This indicates that not just bacteria, but spores as well could be rising to the top.

Gravity Separation of Somatic Cells

The SC are composed mainly of white blood cells (leukocytes), which includes macrophages, lymphocytes, neutrophils, and a few epithelial cells (Kehrli, Jr. and Shuster, 1994). The SC present in milk are still viable, with around 60-75% viable (Verdi and Barbano, 1988; Baumert et al, 2009). In healthy cows, macrophages and lymphocyctes make up the majority of SC in milk. In cows with mastitis, neutrophils become the dominant type of SC in milk. The increase in SC during mastitis is an immune response to pathogens in the mammary gland (Kehril, Jr. and Shuster, 1994).

Very little research has looked at the gravity separation of somatic cells (SC) in milk. It has been discovered that along with fat and bacteria, SC gravity separates as well (Caplan et al., 2013). Similar to the rising of bacteria and fat, SC would concentrate in the top portion of the milk after gravity separation. Caplan et al. (2013) also found a similar trend among the gravity separation of fat, bacteria, and SC, however SC and bacteria were more concentrated after 22 hours at 4°C than the fat. The role of SC in the gravity separation of fat and bacteria is uncertain, as its unknown at this time if it's a necessary component.

Cottage Cheese Culture Agglutination.

A curious phenomenon found in cottage cheese is the agglutination of starter cultures into clumps that settle at the bottom of the cheese vat. This causes the acid production to decrease and the curds to shatter more easily (Emmons et al., 1966). The agglutination of the culture varies based on the type of bacteria species used, indicating an antibody is responsible for the agglutination (Emmons et al., 1966).

Further study of the agglutination of cottage cheese cultures shows that heating (Emmons et al., 1966) or homogenizing (Russell-Campbell and Hicks, 1992) the milk reduces the agglutination of cultures. Agglutination also increased if milk had high amounts of colostral or mastitic secretions (Scheuble et al., 1981). Kanno et al. (1976) identified IgM as the factor that caused the culture to agglutinate, however Salih and Sandine (1984) identified IgG as the possible agglutination factor. The amount of Ig needed to agglutinate bacteria were very low (Scheuble et al., 1981), which may also explain why agglutination of bacteria increase when milk is higher in colostral or mastitis secretions, which increase the Ig content of the milk.

The question still remains as to why cottage cheese cultures sink to the bottom as oppose to rise to the top as the normal bacteria found in milk does. The agglutination of starter cultures in cottage cheese appears to have the same mechanism as the rising of fat globules and bacteria during the normal creaming of milk. It may be the particular strains that are used in cottage cheese cultures that cause those particular cultures to sink.

Possible Mechanism for Gravity Separation

Bovine Ig Structure. Since Ig have been identified as the likely heat-labile component of gravity separation, it would prove beneficial to look closer at the properties of Ig to help understand the mechanism behind the gravity separation of fat, bacteria, spores, and SC in milk. Ig are a type of B-cell receptor that binds to antigens. The basic structure of an Ig consists of a Y like structure that has 2 identical heavy chains and 2 identical light chains. The heavy chains are bigger in size than the light chain (Parham, 2009). The molecule also contains a constant region and variable region. There are 2 identical antigen-binding sides on the variable region. The variable region is what gives the Ig the specificity to react with different antigens (Parham, 2009). The Ig molecule is held together by 2 disulfide bonds in the hinge. When the disulfide bond of the hinge is broken, the molecule breaks into 3 fragments: 2 identical fragment antigen binding (FAC) pieces and 1 fragment crystallizable (FC) pieces (Parham, 2009). There is also a disulfide bond linking together the heavy and light chain in both Fab fragments (Parham, 2009).

There are five main classes of Ig: IgA, IgD, IgE, IgG, and IgM. Subclasses exist in some of these classes (Parham, 2009). Slight structural differences in the basic Y molecule cause differences in function of the Ig (Parham, 2009). Within a class of Ig, they contain the same constant region within the heavy chain and it's the differences in these constant regions that define the 5 classes (Parham, 2009). In addition, more than on Y molecule can be linked together. Multiple Y molecules are covalently linked together, and are usually like together by what's called a joining (J) chain (Hurley and Theil, 2011). IgM exists almost always as a pentamer, or 5 molecules bond together. IgA can exist as either a monomer or dimer. IgD, IgE, and IgG exist almost exclusively as monomers (Parham, 2009).

IgM is the antibody involved in the first immune response when an antigen is detected, and therefore has lower specificity (Hurley and Theil, 2011). IgG is involved in the secondary immune response and has more specificity towards the particular antigens (Hurley and Theil, 2011). IgA is the primary antibody found in mucosal secretions (Hurley and Theil, 2011). IgE is responsible for triggering inflammatory and other physical reactions towards antigens and is also

responsible for allergic reactions (Parham, 2009). IgD is found in the serum in low amounts but its role is still uncertain (Parham, 2009).

In human and animals, Ig are secreted from the body into the milk. The composition of Ig in the milk from various specifies varies based on how immunity is passed onto the offspring (through the placenta vs. mammary secretions) (Hurley and Theil, 2011). Colostrum is the first milk obtained after calving and is high in Ig. The Ig content in colostrum decreases overtime until it reaches the normal level found in milk (Hurley and Theil, 2011). For bovines, the calf is borne with little immunity and can absorb Ig from the milk during the first 24-36 hours of life. Colostrum is essential for newborn calves to provide them with the appropriate immunity (Hurley and Theil, 2011).

Bovine milk and colostrum Ig are composed primarily of IgA, IgG1, IgG2, and IgM. The structure of bovine Ig are similar to human Ig, and therefore bovine Ig should behave similar to human Ig (Kumar and Mikolajcik. 1973; Butler, 1983). IgE and IgD are only present in very small amounts. IgE wasn't identified in bovine milk until 1971 (Hammer et al., 1971), although the full role of IgE is uncertain (Gershwin, 2009). IgD is also present in bovine milk, but there is very little research on it (Zhao et al., 2002). The majority of literature does not mention IgE and IgD when talking about bovine Ig. IgE and IgD will not be included in further discussion of bovine Ig due to lack of available information. The average concentration of each Ig in milk and colostrum are shown in Table 1.1. While colostrum contains much higher levels of Ig than in milk, the overall composition remains roughly the same. IgG1 is found in the greatest concentration (Butler, 1983).

Table 1.1. Immunoglobulin composition of bovine colostrum and milk[1]

		IgG1	IgG2	IgA	IgM
Colostrum	Average (mg/mL)	46.4	2.87	5.36	6.77
	Range (mg/mL)	30.0 – 75.0	1.9 – 4.0	1.8 – 14.5	3.2 – 12.1
	Percent of Total	75.6 %	4.7	8.7	11.0
Milk	Average (mg/mL)	0.58	0.055	0.081	0.086
	Range (mg/mL)	0.33 – 1.2	0.037 – 0.06	0.05 – 0.11	0.037 – 0.15
	Percent of Total	72.3%	6.9	10.1	10.7

[1]Values obtained from Butler, 1983.

As shown in Table 1.1, there exists a large variability in the values obtained for the different Ig concentrations. There has been evidence of breed variations in Ig content of colostrum (Kruse, 1970; Muller and Ellinger, 1981). There is conflicting evidence as to whether the season affects the Ig concentration in colostrum: Shearer et al. (1992) found an increase of Ig during summer months. Nardone et al. (1997) found cows under heat stress produced less Ig in colostrum. Kruse (1970) found no seasonal effect on Ig concentration in colostrum.

The Ig concentration of colostrum declines quickly, with decreases seen at least 3 - 6 hours after calving until about 5 days where it reaches the normal composition of milk (Porter, 1971; Stott et al., 1981; Abd El-Fattah et al., 2012). Cows that have had more lactations (greater than 3 or 4) had higher concentrations of total Ig than cows that had only 1 or 2 lactations (Muller and Ellinger, 1981; Gnomes et. al, 2011). When looking at how the specific Ig changed with increasing lactations there were conflicting results between researchers, but it is clear that the number of lactations does have a significant effect (Devery-Pocius and Larson, 1983; Quigley et al., 1994). Devery-Pocius and Larson (1983) speculated that at least part of the increase of Ig with the increase of lactations had to do with the age of the cow, since an older

cow would have been exposed to more antigens. Serum Ig and colostral Ig did not show any correlation (Blecha et al., 1981).

Similarly, there were variations found in the Ig content of milk. The stage of lactation affects the Ig content of milk, with a drop of Ig concentration right after calving, with a slight increase in Ig concentration at the end of lactation due to a decrease in milk yield (Larson and Kendall, 1957; Guidry et al., 1980; Guidry and Miller, 1986). There is also evidence that the number of lactations affects the Ig concentration of the milk (Zhao et al., 2010). All these factors will affect the measured concentration of Ig in milk and colostrum.

Possible Role of Ig in Gravity Separation. One possible mechanism of Ig binding to the MFGM is through the polymeric immunoglobulin receptor (pIgR). Ig that contain J-chains (IgA and IgM) have a high affinity for pIgR (Hurley and Theil, 2011). The pIgR is a receptor found on epithelial cells that transports IgA and IgM (when in their dimer and pentamer form) across the membrane by forming a disulfide bond with the J-chain (Parham, 2009). The pIgR is composed of domains similar to Ig (Parham, 2009). There is evidence that pIgR is found in the MFGM (Murgiano et al., 2009) of bovines. Other IgM receptors have not been well studied, although more recent evidence shows there are receptors for IgM in humans (Kubagawa et al., 2009; Klimovich, 2011). IgG and IgA both have Fc receptors (Monteiro and van de Winkel, 2003; Klimovich, 2011).

IgM participates in the classical pathway of complement system. It has been well studied in humans. The complement system is an immune response consisting of several components that identify and destroy pathogens (Parham, 2009). IgM first binds to the pathogen surface (Parham, 2009). The complement component 1 (C1), q subcomponent (C1q component) of C1 binds at multiple sites to IgM. This starts a chain reaction of activation (Parham, 2009). Complement component 1, r subcomponent (Clr) is activated, which activates, s subcomponent (Cls), which in turn activates complement component 4 (C4) and complement component 2 (C2), causing them both to cleave into fragments C4a and C4b and C2a and C2 b, respectively. The C4b and C2a fragments bind together to form the classical complement component 3 (C3)

convertase (Parham, 2009). The classical covertase C3 then cleaves C3 into C3a and C3b, which allows C3b molecules to bind to the pathogen. Complement receptor 1 (CR1) on the C3b molecules allow the pathogen to be phagocytized (Parham, 2009). IgG can activate the complement system in a similar way, by binding to an antigen and then Clq binding to the Ig molecule thus starting the activation pathway (Parham, 2009). Due to structural similarities between human and bovine Ig, it is likely that in the bovine complement system, IgG and IgM behave in similar manners (Butler, 1983).

Studies on the bovine complement system have shown a type of conglutinin that can cause agglutination (Sage et al., 1963). Bovine milk was found to contain most of the components of the complement system, however the levels of Clq seem too low for the classical pathway to take place, although the alternative pathway has been found in milk which Ig don't participate in (Rainard, 2003). There is evidence of the antibody-complement system in colostrum from bovines and of possible bacterial inhibitors in bovine milk (Korhonen et al., 1995; Reiter and Oram, 1967).

It's unclear on whether the complement system plays a role in the gravity separation. It is clear that IgM can cause agglutination of bacteria through antigen-recognition sites on the bacteria (Klimovich, 2011). IgM has much greater agglutination ability than IgG due to its large size and greater number of antigen binding sites (Klimovich, 2011). IgM that is bound to an antigen can also bind to a B-cell in order to activate it (Parham, 2009). This shows us that when IgM is bound to an antigen, it can also bind to another molecule using a different receptor. Some research has shown that the addition of Ig increase the bacteria bound to fat globules (Stadhouders and Hup, 1970). Perhaps IgM facilitates gravity separation of bacteria by binding to an antigen and then binding to a receptor on the MFGM. Since IgM has several binding sites, there exists the potential for large aggregates of bacteria bound to the MFGM.

HEAT INACTIVATION OF GRAVITY SEPARATION

Heat Inactivation of the Gravity Separation of Fat, Bacteria, Spores, and SC

Little research has looked at the effect of heat on the combined gravity separation of fat, bacteria, spores, and SC, although the evidence suggests a common mechanism. It was noticed that the temperatures used to stop gravity separation of fat were very similar to temperatures that stopped agglutination of bacteria in blood serum (Dunkley and Sommer, 1944). Wright and Tramer (1957) saw the activity of cottage cheese cultures increase along with a decrease in the gravity separation of fat between 73°C and 76°C.

Caplan et al. (2013) found that heat inactivation stops the gravity separation of fat, bacteria, and SC. Heating the milk to >76.9°C for 25s stopped the gravity separation of all three components, further evidence that a common mechanism is used for the gravity separation of fat, bacteria, and SC (Caplan et al, 2013). There has been little research found that looks at the heat inactivation of the gravity separation of spores, but since they appear to gravity separate in a manner similar to bacteria, it suggests that heat should stop their gravity separation as well. There is a strong possibility that Ig could be responsible for the gravity separation of not just fat, but bacteria, spores, and SC.

Ig Denaturation

Mainer et al. (1997) examined the denaturation of bovine IgA, IgG, and IgM in the temperature range of 62 to 81°C. The order of denaturation was IgM, IgA, and IgG, with IgG being the most heat stable out of the three. At 72°C, the D values for IgM, IgA, and IgG were 3 mins, 35 mins, and 58 mins, respectively. The z-values for IgG, IgA, and IgM were 6.29, 4.00, and 5.17°C, respectively (Mainer et al., 1997). For the three Ig, the longer the holding time at each temperature, the greater the degree of denaturation (Mainer et al., 1997). Very little other literature data has looked at the denaturation of bovine IgA and IgM.

However, a lot of research has looked at the heat denaturation of bovine IgG. Variability exists in the reported D-values for IgG, most likely due to a factors like the medium used for heating IgG, the method for isolating IgG, and how IgG activity was determined (Fukumoto el

al., 1994; Li-Chan et al., 1995; Domínguez et al., 1997; Mainer et al., 1997; Mainer et al., 1999; Chen et al., 2000). All the D-values support the idea that temperatures over 72°C will start to have substantial IgG denaturation, given the appropriate holding time. At high temperatures (75°C to 100°C), when IgG is in colostrum, it is more heat stable than when it is in colostral whey or a phosphate buffer saline solution (Chen and Chang, 1998). Milk appears to provide some protection for IgG denaturation as well as added sugars (Chen and Chang, 1998). The pH affects the IgG heat denaturing, with denaturation increasing at lower pH (Domínguez et al., 2001).

In general, there are two main ways to measure the D-value: measuring the immunoreactivity of the whole Ig molecule during heating or measuring the binding of the Ig to specific antigens during heating. The IgG molecule is composed of one Fc and 2 Fab fragments. The Fab fragments are more heat sensitive than the Fc fragments, and will therefore be affected by the heat treatments first (Vermeer and Norde, 2000). When the Fab and Fc fragments heat denature, they form irreversible aggregates (Vermeer and Norde, 2000). More specifically, within the Fab fragments, the CH1 region degrades first (Rotterman et al., 1994). The Fab fragment will begin to denature at 61°C, yet the Fc fragment does not denature until 71°C, which also shows that Fab and Fc can behave independently (Vermeer et al., 2000). Since the antigen-binding site is found on the Fab fragments, then it's reasonable to assume that the binding of the Ig to specific antigens would decrease at lower temperatures than the immunoreactivity of the molecule as a whole (Domínguez et al., 2001).

The research by Vermeer and Norde (2000) and Vermeer et al. (2000) were done using mice IgG. The research by Rotterman et al. (1994) was performed using human IgG. Research by Indyk et al. (2008) suggests than the Fab and Fc portions of bovine IgG can act independently and showed that Fc was structural stabile. This suggests that the research results on the thermal denaturation of IgG in mice and humans may be similar to the thermal denaturation of bovine IgG.

Further research on bovine IgG showed the molecule as a whole will begin to lose its secondary structure at higher temperatures (>72°C), which is an irreversible reaction that causes the IgG to lose its immunoactivity (Li et al., 2005). The secondary structure of β-sheets will begin to form random coils (Li et al., 2005). It's possible the conformation change takes place at either the hinge region of the Ig molecule which connects the 2 Fab fragments with the Fc fragment or in the interdomain of the Fab fragments connecting the constant and variable regions (Calmettes et al., 1991). Both of these regions contain disulfide bonds. The precise mechanism for the denaturation of bovine IgG is still unknown.

It is possible that IgM has similar heat denaturation as IgG. Since IgM is composed of a similar basic Y structure as IgG and usually has 5 Y molecules bond together, there are a lot of disulfide bonds that could be denatured by heat (Parham, 2009). For each Y molecule, there are 2 Fab fragments and each Fab fragment is held together by at least one disulfide bond (Parham, 2009). If the Fab fragments become denatured by heat, the molecule would lose its antigen binding activity (Calmettes et al., 1991). Mainer et al. (1997) showed that IgM is less heat stabile than IgG, but it is still unclear as to why. Euber and Brunner (1984) showed it was the Fab portion of IgM that was responsible for agglutinating fat molecules. One study on human IgM showed the Fab fragment was more heat stable than the Fc fragment, with the Fab fragment denaturing around 73°C and the Fc fragment denaturing around 71°C (Protasevich et al., 2007). The authors also found that the IgM molecule as a whole started to denature at a lower temperature (63°C) than the individual Fab and Fc fragments (Protasevich et al., 2007).

The temperatures found to denature Ig are similar to the temperatures required to stop the gravity separation of fat, bacteria, and SC in milk, giving further evidence that Ig are the heat-labile component. It's uncertain whether IgM is also responsible for the gravity separation of bacteria, spores, and SC or if another Ig(s) is also involved.

RESEARCH OBJECTIVES

Some questions remain on the gravity separation process. Is fat necessary for the gravity separation of SC? Does HTP stop gravity separation in skim milk? Are Ig important in the gravity separation of SC in skim milk? The first research objective was to determine if Ig play a role in the gravity separation of somatic cells in skim milk

Why do bacteria and spores rise to the top instead of sinking to the bottom? What other components are necessary for the gravity separation of bacteria and spores? Do SC play an important role in the gravity separation process? The second research objective was to determine the role that Ig and SC play in the gravity separation of bacteria and spores in whole milk.

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CHAPTER TWO

Impact of Colostrum on Gravity Separation of Milk Somatic Cells in Skim Milk

ABSTRACT

Our objective was to determine if Ig play a role in the gravity separation (rising to the top) of somatic cells (SC) in skim milk. Other researchers have shown that gravity separation of milk fat globules is enhanced by IgM. Our recent research found that bacteria and SC gravity separate in both raw whole and skim milk and that heating milk to >76.9^{o}C for 25s stopped gravity separation of milk fat, SC, and bacteria. Bovine colostrum is a good natural source of Ig. An experiment was designed where skim milk was heated at high temperatures (76°C for 7 min) to stop the gravity separation of SC and then colostrum was added back to try to restore the gravity separation of SC in increasing increments to achieve 0, 0.4, 0.8, 2.0, and 4.0 g/L of added Ig. The milk was allowed to gravity separate for 22 hrs at 4°C. The heat treatment (76°C for 7 min) of skim milk was sufficient to stop the gravity separation of SC. The treatment of 4.0 g/L of added Ig was successful in restoring the gravity separation of SC as compared to raw skim. Preliminary spore data on the third replicate suggested that bacterial spores gravity separate the same way as the SC in heated skim (76°C for 7 min) with 4.0 g/L of added Ig. There is strong evidence that Ig are at least one of the factors necessary for the gravity separation of SC and bacterial spores. It is uncertain at this time whether SC are a necessary component for gravity separation of fat, bacteria and spores to occur. Further research will need to examine separately the role of Ig and SC in gravity separation of bacteria and spores. Understanding the mechanism of gravity separation may allow develop a continuous flow technology to remove SC, bacteria, and spores from milk.

INTRODUCTION

A well-known phenomenon in bovine milk is the gravity separation, or rising to the top, of fat globules when milk is allowed to stand unagitated. It was determined that the fat globules rose quicker than predicted by Stokes' Law (Troy and Sharp, 1928). If the fat globules clustered

together, the faster rising could be explained (Troy and Sharp, 1928). Fat globules in milk aggregate together in a similar fashion to aggregation of components in blood (Babcock 1889).

Bacteria rise to the top during gravity separation along with the fat. Several researchers looked at the composition of the bacteria species before and after gravity separation and found that some species of bacteria rose to the top more than others (Dellaglio et al., 1969; Abo-Elnaga et al., 1981; Franciosi et al., 2011). It's been estimated that between 67 to 99% of the initial bacteria rises to the cream layer after gravity separation (Abo-Elnaga et al., 1981).

There is also competition between bacteria and fat, as milk that is higher in bacteria counts will have a higher percentage of total bacteria at the top but a lower percent of total fat at the top compared to a milk with a lower starting bacteria count (Caplan et al., 2013). Stadhouders and Hup (1970) showed that bacteria agglutinate in milk and become attached to the fat globules, suggesting a similar agglutinating process for fat and bacteria. There is also evidence that spores rise to the top during gravity separation (Rossi, 1964; Dellaglio et al., 1969)

Very little research has looked at the gravity separation of somatic cells (SC) in milk. Similar to the rising of bacteria and fat, SC would concentrate in the top portion of the milk after gravity separation (Caplan et al., 2013). Caplan et al. (2013) also found a similar trend among the gravity separation of fat, bacteria, and SC, however SC and bacteria were more concentrated after 22 hours at 4°C than the fat. The role of SC in the gravity separation of fat and bacteria is uncertain, as it's unknown at this time if it's a necessary component.

What still is not well understood is the actual mechanism behind the gravity separation. In a series of research experiments by Mertens (1933a,b), he came up with two main points regarding the gravity separation of fat. The first is that there is a factor that affects gravity separation in skim milk that can be heat denatured. The second factor is that something in colostrum enhances gravity separation of milk. Previous work showed that pasteurization at temperatures >76.9°C for 25s prevented gravity separation of fat, bacteria and somatic cells in whole milk (Caplan et al., 2013), which agrees with results from Mertens (1933a). Furthermore,

the fact that fat, bacteria, and SC all stop their gravity separation with heat (>76.9°C for 25s) is further evidence of a common mechanism.

Immunoglobulins are a good candidate as the heat-labile component of the gravity separation of fat based on the fact that they are found in blood serum, colostrum, and milk of bovines and they have the capability to form agglutinations (Hurley and Theil, 2011). Colostrum is a rich source of Ig, which could explain why Mertens (1933b) found the addition of colostrum increased the rate of gravity separation. Euber and Brunner (1984) showed that IgM was a necessary component for the gravity separation of fat. The addition of IgM to whole milk heated at 75°C for 30 mins restored the creaming of fat, while the addition of IgG did not (Euber and Brunner, 1984). They also found that when they removed IgA and IgG from raw milk, there was no effect on the creaming but removal of IgM did reduce creaming (Euber and Brunner, 1984).

Immunoglobulins are a type of B-cell receptor that binds to antigens. The basic structure of an Ig consists of a Y like structure that has 2 identical heavy chains and 2 identical light chains (Parham, 2009). The Ig molecule is held together by 2 disulfide bonds in the hinge. There is also a disulfide bond linking together the heavy and light chain (Parham, 2009). It's possible that disulfide bonds in the Ig molecule are destroyed by heat, leading to conformational changes within the Ig molecule causing it to lose it activity. Bovine milk and colostrum Ig are composed primarily of IgA, IgG1, IgG2, and IgM.

There are 2 identical antigen-binding sites found on each Y-structure of the Ig molecule (Parham, 2009). The antigen-binding sites recognize carbohydrates or proteins on the surface of a pathogen (Parham, 2009). There is variability between different antigen-binding sites, which determines which type of antigens it can bind to (Parham, 2009). IgM is involved in the first immune response when an antigen is detected, and therefore has lower specificity (Hurley and Theil, 2011). IgG is involved in the secondary immune response and has more specificity towards particular antigens (Hurley and Theil, 2011). IgA is the primary antigen found in mucosal secretions (Hurley and Theil, 2011).

Research in our lab has shown that SC will gravity separate in skim milk, in the absence of fat globules. There is evidence (Euber and Brunner, 1984) that milk fat globule membrane (MFGM) material may be important in the gravity separation process, not the actual fat globules themselves. It has been observed that homogenization will stop the gravity separation of fat milk. Euber and Brunner (1984) added IgM and skim milk membrane separately to homogenized milk. The addition of IgM had no effect on the gravity separation of fat, but the addition of the skim milk membrane restored the gravity separation. MFGM material is found in both whole milk and skim milk, which could explain why somatic cells gravity separate in both skim and whole milks.

There was little research found comparing the concentrations of Igs in whole milk vs. skim, but it appears that at least for IgG, there was no change in concentration after the cream was removed (Li-Chan et al., 1995). While no literature found compares IgM and IgA in whole vs. skim, there is a higher association of IgM and IgA with fat than for IgG1 and IgG2 (Frenyo et al., 1986). It is possible therefore that the levels of IgM and IgA would be lower in skim. However, Euber and Brunner (1984) found that only 7% of the total amount of IgM present in whole milk is needed for gravity separation of fat. It's likely then that even if the Ig levels are lower in skim compared to whole, it's not enough to prevent the gravity separation of SC in skim milk.

More research is needed to understand how SC gravity separate in skim milk and whether SC plays an important part in the gravity separation process. Our objective was to determine if Ig play a role in the gravity separation of SC in skim milk. Bovine colostrum served as a source of Ig, due to the high concentration of Ig as compared to milk.

MATERIALS AND METHODS

Experimental Design and Statistical Analysis

An experiment was designed to determine if addition of raw colostrum (a crude source of bovine Ig) could restore gravity separation of somatic cells for pasteurized (>74.5°C for 25s) skim milk. There were 6 treatments: raw skim, pasteurized skim and pasteurized skim with 4

levels of added Ig from colostrum (0.4, 0.8, 2.0, and 4.0 g/L of added Ig). Six fractions were collected by weight starting from the bottom of each gravity separation column (0 to 90%, 90 to 92%, 92 to 94%, 94 to 96%, 96 to 98%, and 98 to 100%) after 22 h at 4°C. The SCC was measured in the 6 fractions for each of the columns. The experiment was replicated 3 times in different weeks using a different batch of milk and a different colostrum.

The Proc GLM procedure of SAS (9.3) was used to determine if level of addition of colostrum influenced the starting SCC of the 6 milks in the gravity separation columns. The ANOVA model had terms for level of colostrum addition and replicate as a category variables. A linear regression analysis was done to determine if there was a significant correlation between the amount of colostrum added (i.e., estimated about of Ig added) and the amount of SC in the top 2% layer.

Collection and Analysis of Colostrum

Colostrum was obtained from Holstein cows at the Cornell University Teaching and Research Center on the first day of calving. The colostrum was placed on ice in a cooler held at 4°C until ready to use. The protein and fat level of the colostrum was determined using a Fourier transform mid-infrared transmittance milk analyzer (LactoScope FTIR Advanced (FTA), Delta Instruments, De Boulder 68, The Netherlands). The calibration of the infrared milk analyzer was done as described by Kaylegian et al. (2006a,b) using a set of modified milk calibration samples. It was assumed that all the true protein in the colostrum higher than a concentration of 3% protein, the typical true protein level of milk, was due to immunoglobulins. This estimate of total immunoglobulin content was used to estimate the grams per liter of immunoglobulin each colostrum. Fresh colostrum was obtained for each replicate of the experiment.

Milk Processing

Raw whole bulk tank milk was collected from Holstein cows at the Cornell University Teaching and Research Center. The milk was mixed and separated at 4^{o}C using a cream separator (Model 372 Airtight, DeLaval Separator Co., Poughkeepsie, NY). One portion of the raw milk was held at 4°C and another portion of the raw skim milk (about 20 kg) was batch

pasteurized at 76°C for 7 min using a 38 L stainless steel jacketed steam kettle. Previous research (Caplan et al., 2013) has demonstrated that this heat treatment will prevent gravity separation. After 7 min, about 17 L of pasteurized skim was collected and rapidly cooled in ice water and placed in a cooler at 4^{o}C.

Six one-liter glass gravity separation columns with a Teflon stopcock at the bottom (cat no. 03-789-5c, Fisher Scientific, Pittsburg, PA) had been rinsed 3 times with a 200 ppm chlorine sanitizer solution and 3 times with a 70% ethanol solution the day before the experiment. Each column outlet was sanitized and had a sanitized Pasteur pipette bulb (cat no. 03-448-22, Fisher Scientific) on the bottom outlet to close the open glass tube with the stopcock closed and the top of the column capped with a rubber stopper. The empty columns were placed into a 4°C cooler. After pasteurization and cooling of the skim milk, one glass column was filled with raw skim and the other 5 were filled with pasteurized skim containing increasing amounts of added colostrum. The approximate weight of colostrum added was designed to provide about 0, 0.4, 0.8, 2.0, or 4.0 g/L of added Ig to approximately 1000 mL of milk in the gravity separation column. The milk and colostrum were added to the columns by weight. To ensure thorough mixing of the milk and colostrum prior to addition to the glass column, approximately 1,050 g of milk were added to a sanitized plastic 5.7 L container and the appropriate weight of colostrum was added. The milk plus colostrum was mixed thoroughly, about 50 g was poured into a 60 mL sterile plastic snap-top vial (cat no. CPP03CL, Capital Vial Inc., Amsterdam, NY) for analysis, and the rest was poured into the glass column and the weight of milk in each column was recorded. The top of the columns were closed with stopper and immediately placed into a 4°C cooler and the milk was allowed to gravity separate for 22:40 h ± 3.1 min.

Sampling and Analysis of Milk Fractions

The fat and SCC concentrations were measured for the raw whole milk, raw cream, colostrum and the milk plus colostrum for each of the 6 columns. Six fractions were collected from each of the columns. The milk was collected by weight, starting from the bottom of the column. The bottom 90% was collected by weight into a sanitized plastic 1.9 L container**.** The

top 10% layer was collected in 5 equal portions by weight directly into 45 mL sterile plastic snap-top vials (cat no. CPP02CL, Capital Vial Inc., Amsterdam, NY). The fractions collected were placed immediately into a 4°C cooler. Each fraction was analyzed for SCC. Fractions outside of the range of the instrument calibration (i.e., > 1,000,000 SCC/mL) were diluted (w/w) using raw cold separated skim. A dilution factor was used to calculate the SCC of the undiluted fraction. The cream and colostrum were also diluted with skim milk before SCC analysis to reduce their viscosity and obtain a concentration of SC within the instrument's calibration range.

The fat content was determined (AOAC 2000: method 972.16) using a Fourier transform infrared dairy analyzer (LactoScope FTIR, Delta Instruments, De Boulder 68, The Netherlands). The SCC was determined (AOAC 2000: method 17.13.01; 978.26) using fluorescence flow cytometry (SomaScope, Delta Instruments, De Boulder 68, The Netherlands).

RESULTS AND DISCUSSION

Composition and SCC of Whole Milk and Colostrum

The SCC for the whole milk used in each replicate is shown in Table 2.1.The mean whole milk SCC of 241,000 SCC/mL used in this study represents a typical bulk raw milk from the Cornell University dairy herd. For bulk tank milk, the USDA Federal Milk Markets has a milk quality payment system based on SCC in selected federal orders (USDA Federal Milk Market, 2012). Farms producing milk with SCC <350,000 cells/mL receive more for their milk, while those with > 350,000 cells/mL receive a lower price for their milk. The mean SCC in colostrum (Table 2.1) was much higher than raw whole (3,799,000/mL vs 241,000/mL). The range of SC in colostrum was consistent with the findings of other researchers. In a sample of 69 Holstein cows, Ferdowsi Nia et al. (2010) found a range of 641,000 to 9,620,000 cells/mL for colostral SC.

Table 2.1. Somatic cell count (SCC) per mL for the raw whole milk and colostrum for each replicate. Mean ± one standard deviation.

Starting Materials	Replicate	SCC/mL
Raw Whole	1	215,000
	2	256,000
	3	252,000
	Mean	241,000 ± 23,000
Colostrum	1	3,905,000
	2	5,428,000
	3	2,053,000
	Mean	3,795,000 ± 1,690,000

The percent true protein, percent fat, and age of the colostrum for each replicate are shown in Table 2.2. The amount of true protein and fat varied among the 3 colostrums. Moody et al. (1951) also found that the protein and fat varied considerably in colostrum, with fat ranging from approximately 1% to almost 14% and protein ranging from about 9% to over 20%. Our ranges in Table 2.2 are consistent with their findings.

Table 2.2. The percent of true protein and fat in the colostrum and the age of the colostrum at time of experiment.

Replicate	True protein (%)	Fat (%)	Age of colostrum (days)
1	22.1	9.64	4.7
2	16.1	3.84	2.9
3	15.4	3.60	5.0

We accounted for differences in the protein content among colostrums to estimate the total amount of Ig that was added to each gravity separation column for each replicate. We assumed that all protein over 3%, the normal protein level of milk, was Ig. There could have been differences in the proportions of different types of Ig from one experimental replicate to another among the 3 different colostrums. The relative proportion of the different types of individual Ig in the colostrums used in this study was not measured.

The fat content also varied among the 3 colostrums (Table 2.2). The fat content of colostrum was much higher for the first replicate than the other 2 replicates. We did not account for variation in fat when adding colostrum to the skim. The age of the colostrum at the time of the experiment differed slightly among replicates, varying between 2.9 and 5 days, depending on the day the cow had its calf. The colostrum was collected on the first day of calving for all replicates. The colostrum for each replicate was held on ice in a 4°C cooler prior to the each replicate.

Starting SCC of Skim Milks in the Gravity Separation Columns.

The initial SCC/mL and the amount of colostrum added (g) to achieve the target Ig addition for each treatment and replicate are shown in Table 2.3. There was an effect ($P < 0.05$) of added colostrum and replicate on SCC of the milk plus colostrum mixture with SCC increasing linearly ($P < 0.05$) as the amount of added colostrum increased (R-square 0.895). This was expected because adding more colostrum would also add more SC. Variability in starting milk SCC/mL in the columns (Table 2.3) among reps was expected because differences in SCC among the 3 different colostrums (Table 2.2). The amount of colostrum added to each treatment (Table 2.3) varied from one replicate to another due to differences in the amount of protein from one colostrum to another (Table 2.2).

Table 2.3. The initial somatic cell count (SCC) per mL, the amount of colostrum added (g/1000 g milk), and the percent of SC in the top 2% layer after gravity separation for 22h at 4°C for the raw skim and the 5 pasteurized skim milk treatments of added colostrum (0, 0.4, 0.8, 2.0 and 4.0 g/L of added Ig).

Treatment	Colostrum added (g/1000 g milk)[1]	Estimated Ig (g/L) Added	SCC/mL[2]	SC in top 2% layer (%)[3]
Raw skim	-	-	94,000[c]	81.5[a]
Pasteurized skim	0[e]	0	94,000[c]	2.2[c]
Pasteurized skim	2.77[d]	0.4	110,000[b,c]	2.4[c]
Pasteurized skim	5.64[c]	0.8	117,000[b,c]	2.2[c]
Pasteurized skim	14.20[b]	2.0	149,000[b]	36.3[b]
Pasteurized skim	31.25[a]	4.0	215,000[a]	77.2[a]
R-Square	0.992		0.895	0.960

[a-e] Means within a column with different subscripts differ ($P < 0.05$).
[1]Standard error for of colostrum added for the pasteurized skim with 0, 0.4, 0.8, and 2.0 g/L added Ig was 0.77 and 0.98 for 4.0 g/L added Ig.
[2]Standard error for initial SCC/mL for the raw skim and pasteurized skim with 0, 0.4, 0.8, and 2 g/L added Ig was 13,631 and 17,242 for 4.0 g/L added Ig.
[3]Standard error for percent of SC in top 2% layer for the raw skim and pasteurized skim with 0, 0.4, 0.8, and 2 g/L added Ig was 5.5% and 7.0% for 4.0 g/L added Ig.

Impact of Pasteurization of Skim Milk on Gravity Separation of SC

Immunoglobulins in colostrum are likely to be at least partially responsible for the restoration of gravity separation in skim milk that was pasteurized at 75°C for 7 min. The three main Ig in colostrum are IgG, IgM, and IgA. IgG can be subdivided into IgG_1 and IgG_2 (Hurley and Theil, 2011). All Ig share a common basic structure. Immunoglobulins are composed of two identical heavy chains and two identical light chains. Each heavy and light chain contains a variable and constant region (Hurley and Theil, 2011). Disulfide bonds link the heavy chains and light chains together, giving the molecule a Y-shape structure (Hurley and Theil, 2011).

There are differences in the overall structure of the three Ig classes in colostrum. IgG exists as a monomer, IgM exists as a pentamer, and IgA exists as a monomer and a dimer (Hurley and Theil, 2011). A joining chain links the monomeric Ig molecule together using a covalent bond to form the polymeric Ig (Hurley and Theil, 2011). Disulfide bridges are

susceptible to degradation when exposed to high enough temperatures (Hurley and Theil, 2011). A degradation of the disulfide bridges would cause a loss of function of the Ig. Research by Caplan et al. (2013) showed that heating whole milk to above 76.9°C for 25s caused a loss of gravity separation of fat and SC, possibly due to the breakage of the disulfide bonds in Ig.

Mainer et al. (1997) examined the denaturation of IgA, IgG, and IgM in the temperature range of 62 to 81°C. The order of denaturation was IgM, IgA, and IgG, with IgG being the most heat stable out of the three (Mainer et al., 1997). For the three Ig, the longer the holding time at each temperature, the greater the degree of denaturation (Mainer et al., 1997). Based on their results, the time/temperature combination of 75°C for 7 min used in our experiment would result in significant degradation for the three Ig.

Looking at Figure 2.1, the SC rose to the top of the column for the raw skim but did not for the pasteurized skim with no added colostrum. 81.5% and 2.2% of the SC were found in the top 2% layer for the raw skim and pasteurized skim, respectively (Table 2.3). These results showed that the heat treatment used stopped the gravity separation of SC in skim milk, similar to the work performed by Caplan et al. (2013) on whole milk. This gives strong evidence that since Ig are destroyed by heat and heat can stop gravity separation, then Ig play at least some role in the gravity separation of SC in skim milk.

Impact of Colostrum Addition to Pasteurized Skim Milk on Gravity Separation of SC

A graph of the cumulative SC contained in the column after gravity separation as a function of the cumulative mass of milk removed from the bottom of the gravity separation columns for the 5 treatments (0, 0.4, 0.8, 2.0, and 4.0 g/L of added Ig) is shown as Figure 2.1. If there was no gravity separation of somatic cells then about 90% of the cumulative somatic cells would be in the lower 90% of the gravity separation column and this was what was observed for pasteurized skim and pasteurized skim with 0.4 and 0.8 g /L of Ig (Figure 2.1). In contrast, the lower 90% of the raw skim only contained about 10% of the SC originally present in the milk (Figure 2.1) indicating that there was substantial gravity separation (i.e., rising of SC to the top) in raw skim milk. To demonstrate the degree of gravity separation of SC, the percentage of the

starting SC in the milk that was in the top 2% of the weight of the milk in column is shown in Table 2.3. Raw skim had an average of 81.5% of total SC in the top 2% layer after 22 h of gravity separation at 4°C, while no gravity separation of the SC was detected in the same milk that was pasteurized (Figure 2.1) at 75°C for 7 min and this was consistent with the results for whole milk presented previously by (Caplan et al., 2013). No gravity separation of SC for pasteurized skim with 0.4, and 0.8 g/L of added Ig was detected ($P > 0.05$).

The error bars in Figure 2.1 show the variability within treatments between replicates. The treatments with 2.0 and 4.0 g/L of added Ig had the highest standard errors. Presumably, this was due to differences in the Ig content in the protein in colostrum among the 3 different cows used in our study. The amount of Ig added and the differences in the proportion of each type of Ig (e.g., IgG, IgM, and IgA) could affect how well the milk gravity separates.

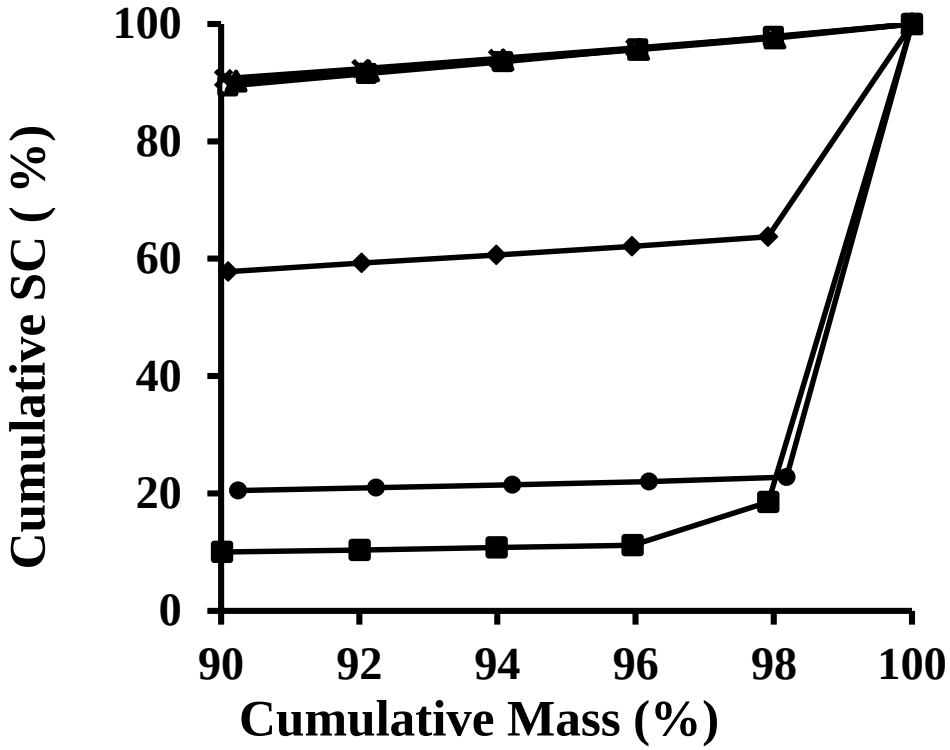

Figure 2.1. A comparison of the mean (n = 3)[a] of the cumulative percent somatic cell count (SCC) accounted for as a function of cumulative mass percent of milk for the 6 treatments, raw (■) and pasteurized skim with 0 (□), 0.4 (▲), 0.8 (×), 2.0 (♦), and 4.0 (●) g/L added Ig, for gravity separation at 4°C for 22h. Error bars represent one standard error from the mean.
[a]Note that for the treatment of 4.0 g/L of added Ig, n = 2

Gravity separation of SC for pasteurized skim with 2.0 and 4.0 g/L of added Ig was compared to the raw skim milk treatment. There was 36 and 77.2% of the SC recovered (for the 2.0 and 4.0g/L of Ig added, respectively) in the top 2% layer compared to 81.5% in the raw skim (Table 2.3). This value for percent of SC in the top 2% layer for the 2.0 g/L addition of Ig was lower ($P < 0.05$) than the raw and 4.0 g/L added Ig, indicating partial restoration of gravity separation. No difference in the percentage of SC in the top 2% between the raw milk and the pasteurized milk with 4.0 g/L of Ig was detected ($P > 0.05$), indicating that 4g/L of added Ig restored the gravity separation of SC in pasteurized skim milk (Table 2.3). This is similar to the results by Jenness and Parkash (1971), who observed that Ig isolated from colostrum and added to pasteurized (85°C for 30 min) whole milk restored the gravity separation of fat.

As the amount of Ig added to pasteurized skim milk increased, the percentage of total SCC that were in top 2% layer after 22 h at 4^{o}C increased linearly, in dose response relationship (Figure 2.2). As mentioned before, colostrum from a different cow was used for each replicate. Cows differ in the properties of their colostrum (Korhonen et al., 2000) and that difference could affect the relative proportion of Ig in the protein. The total Ig content of colostrum can vary considerably, from 30 to 200 mg/mL, between cows due to variations between breeds as differences in age, health status, and lactation stage (Korhonen et al., 2000). Roughly 70 to 80% of the true protein content of colostrum is made up of Ig (Korhonen et al., 2000). While all the colostrum used in this experiment was taken from the cows on the first day of calving, differences in the age and health status as well as time taken after calving, might cause differences in the Ig composition among the 3 colostrum used for this experiment. This could cause variation in observed completeness in gravity separation among replicates for each treatment.

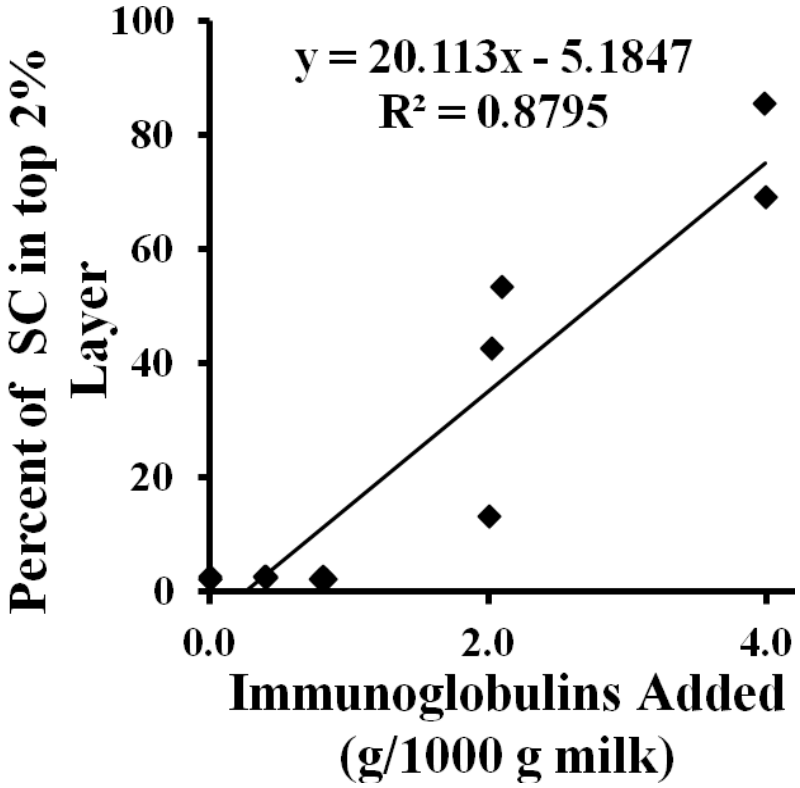

Figure 2.2. The percentage of the total somatic cells (SC) in the top 2% layer of the gravity separation columns for pasteurized skim after 22h at 4°C as a function of the amount of Ig added (g) from the colostrum.

For the last replicate of the experiment, the bacteria spore count was measured for the 0 to 90% and 98 to 100% fractions for the column with raw skim and 3 of the columns with pasteurized skim (0, 2.0, and 4.0 g/L added Ig). The spores gravity separated in fashion similar to the SC. For the raw skim, 80.4% of the spores were found in the top 2% layer. The percent of spores in the top 2% layer were 1.7, 3.8 and 22.6% for the 0, 2.0, and 4.0 g/L of added Ig, respectively. Future work should examine if the gravity separation of vegetative bacterial cells and spores are similar to the gravity separation in SC in skim milk (Figure 2.1) and bacteria in whole milk (Caplan et al., 2013).

CONCLUSIONS

Heat treatment of skim milk (76°C for 7 min) was sufficient to stop the gravity separation of SC. Colostrum was added back to heated skim in increasing increments to achieve 0, 0.4, 0.8, 2.0, and 4.0 g/L of added Ig. The treatment of 4.0 g/L of added Ig was successful in restoring the gravity separation of SC as compared to raw skim. Preliminary spore data on the third replicate suggested that bacterial spores gravity separate the same way as the SC in heated skim and heated skim with 4.0 g/L of added Ig. There is strong evidence that Igs are at least one of the factors necessary for the gravity separation of SC and bacterial spores. It is uncertain at this time whether SC are a necessary component for gravity separation of fat, bacteria and spores to occur. Further research will need to examine separately the role of Ig and SC.

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CHAPTER THREE

The Effect of Ig and Somatic Cells on the Gravity Separation of Fat, Bacteria, and Spores in Pasteurized Whole Milk

ABSTRACT

Our objective was to determine the role that Ig and somatic cells (SC) play in the gravity separation of milk. There were 9 treatments: (1) low temperature pasteurized (**LTP**) (72°C for 17.31s) whole milk, (2) LTP (72°C for 17.31s) whole milk with added bacteria and spores, (3) recombined LTP (72°C for 17.31s) whole milk with added bacteria and spores, (4) high temperature pasteurized (**HTP**) (76°C for 7 min) whole milk with added bacteria and spores, (5) HTP (76°C for 7 min) whole milk with added bacteria and spores and added colostrum, (6) HTP (76°C for 7 min) centrifugal separated gravity separated (**CS GS**) skim milk with HTP (76°C for 7 min) low SC cream with added bacteria and spores, (7) HTP (76°C for 7 min) CS GS skim milk with HTP (76°C for 7 min) high SC cream with added bacteria and spores, (8) HTP (76°C for 7 min) CS GS skim milk with HTP (76°C for 7 min) low SC cream with added bacteria and spores and added colostrum, and (9) HTP (76°C for 7 min) CS GS skim milk with HTP (76°C for 7 min) high SC cream with added bacteria and spores and added colostrum. The milks in 9 treatments were gravity separated at 4°C for 23 h in glass columns. There were 5 fractions collected by weight from each of the columns treatments starting from the bottom of the glass column: 0 to 5%, 5 to 90%, 90 to 96%, 96 to 98%, 98 to 100%. The SC, fat, bacteria, and spores were measured in each of the fractions. The experiment was replicated 3 times in different weeks using a different batch of milk and a different colostrum. Portions of the same batch of the frozen bacteria and spore solutions were used for all 3 replicates. The presence of both SC and Ig were necessary for normal gravity separation (i.e., rising to the top) of fat, bacteria, and spores in whole milk. The presence of Ig alone without somatic cells was not sufficient to cause bacteria, fat and spores to rise to the top without SC. The interaction between SC and Ig was necessary to cause aggregates of fat, SC, bacteria, and spores to rise during gravity separation.

The SC may provide the buoyancy required for the aggregates to rise to the top due to gas within the SC. More research is needed to understand the mechanism of the gravity separation process.

INTRODUCTION

The process of gravity separation has been used for traditional cheese making of Grana and Parmigiano-Reggiano raw milk cheeses in the north of Italy (Fox et al., 2004). The primary purpose of the gravity separation step in the manufacture of these cheeses is to decrease the fat content of the milk prior to cheese making by removal of the upper cream layer (Fox et al., 2004). Traditional Parmigiano-Reggiano cheeses are made by allowing raw milk to gravity separate overnight at 20°C, removing some of the cream layer, and mixing in fresh raw whole milk to obtain a 2.4-2.5% fat milk. The milk for traditional Grana Padano cheeses are prepared by gravity separating raw whole milk at 12-15°C for 12 hours and then removing the fat layer to get a 2.1-2.2% fat milk (Fox et al., 2004).

Euber and Brunner (1984) reported that IgM is involved in the aggregation and gravity separation of milk fat globules. There are two types of lymphocytes in blood: B-cells and T-cells. Immunoglobulins are produced by B cells (Parham, 2009). The B cells produce Ig that are specific to a particular pathogen, based on an encounter of the B cell with that pathogen (Parham, 2009). B cells have Ig bound to the cell membrane exterior and when the bound Ig binds to a pathogen, the B cell is stimulated to produce free Ig molecules to bind to that pathogen. Those Ig produced can only bind to that specific antigen (Parham, 2009).

Immunoglobulins bind to bacteria and spores by recognizing specific carbohydrates or proteins on the outside layer of the pathogen (Parham, 2009). By attaching to the surface of the pathogen, the Ig help signal to other white blood cells the presence of the pathogen (Parham, 2009). Other white blood cells (neutrophil and macrophage) can then destroy the pathogen or activate the complement system (Parham, 2009). The complement system is a series of proteins that are generally proteolytic in character (i.e., serine proteases) in the blood that bind to the surface of bacteria to mark them as targets for phagocytosis by neutrophils and macrophages. This interaction between the complement, Ig, bacteria and spores, and white blood cells in the

body may be involved in the aggregation of bacteria and spores in milk during gravity separation.

Colostrum has a much higher concentration of Ig than milk. Total Ig are present at an average concentration of 61.4 mg/mL in colostrum and 0.8 mg/mL in milk (Butler, 1983). Vaccination of cows can change the Ig composition of the milk. Vaccination against *Escherichia coli* O111:B4, also known as J5 vaccination, caused the milk to contain IgM, IgG1, and IgG2 specific towards J5 (Wilson et al., 2009). Cows that were immunized with inactivated *Staphylococcus aureus* strains had IgA present in milk that was specific for *Staphylococcus aureus* (Tempelmans Plat-Sinnige et al., 2009).

It appears that gravity separation may play an important role in reducing the bacteria and spore content of the milk prior to manufacture of grana style cheeses. Traditional cheese makers have stated that they continue to use gravity separation as opposed to more modern centrifugal separation due to better cheese flavor and fewer gas defects (Caplan et al., 2013). Caplan et al. (2013) reported that fat, SC, and bacteria gravity separate in raw and pasteurized (72.6°C for 25s) whole milk by rising to the top. Both Dellaglio et al., (1969) and Rossi (1964) reported that spores rise to the top during the gravity separation process. The flavor difference when using gravity separation to produce these cheeses may be due to the removal of undesirable bacteria and spores in the cream layer.

The gravity separation of fat, bacteria, and SC in whole milk was stopped when the milk was pasteurized at temperatures >76.9°C for 25s (Caplan et al., 2013). When colostrum was added to pasteurized (76°C for 7 min) skim milk, it restored the gravity separation of SC (Chapter Two). Thus, SC gravity separates even when the fat has been removed from milk. The gravity separation of fat was restored when IgM was added back to whole milk subjected to high heat treatment and when IgM was removed using an IgM specific antiserum, the gravity separation of fat did not occur (Euber and Brunner, 1984). Immunoglobulins are a good candidate for the heat-labile component of the gravity separation of fat based on the fact that they are found in bovine blood serum, colostrum, and milk and they have the capability to form

agglutinations of bacteria (Hurley and Theil, 2011). Our objective was to determine the role that Ig and SC play in the gravity separation of bacteria and spores in milk.

MATERIALS AND METHODS

Experimental Design and Statistical Analysis

An experiment was designed to determine if the presence of SC and/or Ig is necessary for gravity separation to occur in milks. There were 9 treatments (shown in Table 3.1): (1) low temperature pasteurized (**LTP**) (72°C for 17.31s) whole milk, (2) LTP (72°C for 17.31s) whole milk with added bacteria and spores, (3) recombined LTP (72°C for 17.31s) whole milk with added bacteria and spores, (4) high temperature pasteurized (**HTP**) (76°C for 7 min) whole milk with added bacteria and spores, (5) HTP whole milk (76°C for 7 min) with added bacteria and spores and added colostrum, (6) HTP (76°C for 7 min) centrifugal separated gravity separated (**CS GS**) skim milk with HTP (76°C for 7 min) low SC cream with added bacteria and spores, (7) HTP (76°C for 7 min) CS GS skim milk with HTP (76°C for 7 min) high SC cream with added bacteria and spores, (8) HTP (76°C for 7 min) CS GS skim milk with HTP (76°C for 7 min) low SC cream with added bacteria and spores and added colostrum, and (9) HTP (76°C for 7 min) CS GS skim milk with HTP (76°C for 7 min) high SC cream with added bacteria and spores and added colostrum.

The milks in 9 treatments were gravity separated at 4°C for 23 h in glass columns. There were 5 fractions collected by weight from each of the columns treatments starting from the bottom of the glass column: 0 to 5%, 5 to 90%, 90 to 96%, 96 to 98%, 98 to 100%. The SC, fat, bacteria, and spores were measured in each of the fractions. The experiment was replicated 3 times in different weeks using a different batch of milk and a different colostrum. Portions of the same batch of the frozen bacteria and spore solutions were used for all 3 replicates.

The Proc GLM procedure of SAS (9.3) (Cary, NC) was used to determine if the presence or absence of Ig and/or SC caused gravity separation of fat, SC, bacteria, and spores in whole milk. The ANOVA model had terms for treatment and replicate as categorical variables. If the F-test for the model was < 0.05, then the percentage of SC, fat, bacteria, and spores in the different

treatments were compared using H_0: LSMean(i)=LSMean(j) within each fraction in the gravity separation column.

To determine if the gravity separation of SC, fat, bacteria, and spores behave the same way in whole and skim milk, a separate experiment was performed using skim milks: (1) LTP skim milk (72°C for 17.31s) and (2) HTP skim milk (76°C for 7 min). The skim milks were gravity separated for about 22.5 h at 4°C and 4 fractions were collected by weight: 0 to 90%, 90 to 96%, 96 to 98%, 98 to 100%. The data from the 2 skim treatments were analyzed separately from the 9 whole milk treatments. The Proc GLM procedure of SAS (9.3) was used to compare the gravity separation of SC, bacteria, and spores in the 0 to 96% fraction and 96 to 100% fraction in the skim milks. The ANOVA model had terms for treatment, replicate, and replicate*treatment as categorical variables. If the F-test for the model was < 0.05, then the percentage of SC, bacteria, and spores in the different skims were compared using H_0: LSMean(i)=LSMean(j) within each fraction in the gravity separation column.

Collection and Analysis of Colostrum

Colostrum was collected from Holstein cows at the Cornell University Teaching and Research Center from the first milking after calving. The colostrum was held at 4°C after collection and was centrifuged and frozen within 2 days of collection. The colostrum was centrifuged twice using a GSA rotor (Sorval Evolution RC Superspeed Centrifuge, Kendro Laboratory Product, Newtown, Connecticut) at 2000 g for 15 min to remove the pellet of SC and remove the fat as the upper cream layer. The colostrum was centrifuged as opposed to gravity separated to remove the fat (cream layer) and SC (pellet) due to the fact that colostrum with total protein >17% did not gravity separate. The fact that high protein colostrum did not gravity separate is consistent with a report by Moody et al. (1951) that colostrum did not gravity separate when total protein exceeded 16%. The low fat, low SC colostrum was stored at -80°C until ready for use. A separate colostrum sample from a different cow was used for each replicate of the experiment.

The centrifuged colostrum was thawed on the day when the gravity separation experiment was going to be done by first placing in a water bath at room temperature for 2 min. It was then placed in a microwave oven (Kenmore, Model 721.69072, 700 watt, Hoffman Estates, Il) for 10 s at full power, inverted several times to mix, and then repeated at 5s intervals in the microwave until solution reached approximately 9°C. The protein and fat concentrations in the colostrum was determined using a Fourier transform infrared milk analyzer (LactoScope FTIR Advanced (FTA), Delta Instruments, Drachten, The Netherlands). It was assumed that all the true protein in the colostrum above 3% protein, the typical true protein level of milk, was due to Ig. This estimate of total Ig content was used to calculate the grams per liter of Ig in each colostrum. The target final Ig level for the colostrum added to each gravity separation column was 4.0 g/L of Ig. A different colostrum sample was used for each replicate of the experiment.

Preparation of Bacteria

A culture of a previously isolated *Enterococcus faecalis* from unpasteurized milk was stored in 15% glycerol in cryovials (Cat No. 89094-806, VWR North America, Radnor, PA) at -80°C. The *Enterococcus faecalis* culture was streaked on a brain-heart infusion (BHI) plate (BHI Broth - Cat. No. 237500, Becton, Dickinson, and Company, Franklin Lakes, New Jersey; BHI agar - Cat. No. 211065, Becton, Dickinson, and Company, Franklin Lakes, New Jersey) and incubated for 24 h at 32^{o}C. A colony was picked off the BHI plate and a tube of BHI broth was inoculated and incubated at 32°C for 22 h. The BHI broth solution was centrifuged at 13000 rpm for 1 min, the supernatant was discarded and the pellet was mixed into autoclaved skim milk broth (SMB) (Cat. No. 232100, Becton, Dickinson, and Company, Franklin Lakes, New Jersey). A batch of the bacteria in SMB solution (about 40 mL) was made, mixed thoroughly, and divided into 1 mL portions in 1.5 mL safe-lock tubes (Cat. No. 022363204, Eppendorf North America, Hauppauge, NY). The 1 mL portions were placed into a -80°C freezer until ready for use. The same batch of bacteria solution was used for all 3 replicates. Prior to adding the bacteria solution to the milk for a gravity separation column, the 1 mL portion was allowed to thaw at room temperature for approximately 15 min.

Preparation of Spores

Paenibacillus spp. was isolated from raw whole milk in 2006 and stored in 15% glycerol in cryovials at -80°C. The *Paenibacillus spp.* culture was streaked on a BHI plate in three quadrants. The plates were incubated at 32°C for 2 days, after which the plates were checked for individual colony growth and possible contamination. Typical colonies were removed from the plates and streaked onto a new BHI plate. The entire plate was streaked and stored in a 32°C incubator to encourage a large amount of growth. The plates were checked for spores after 2 weeks using the cold method of Schaeffer-Fulton endospore stain (Reynolds et al., 2009). Prior to the experiment, a large batch of spores were produced. The colonies with their spores were scraped off the BHI plate and mixed into autoclaved SMB. The spore SMB solution (about 40 mL) was vortexed and divided into 1 mL portions in 1.5 mL safe-lock tubes. The 1 mL solutions were placed into a -80°C freezer. The vegetative cells were not destroyed, so the final spore solution contained a mixture of endospores and their vegetative cells. Prior to adding the spore solution to the milk, the solution was allowed to thaw at room temperature for approximately 15 min.

Milk Processing and Sampling of Fractions

Day 1. On day 1 of the experiment, raw whole milk was obtained from Holstein cows at Cornell University Teaching and Research Center. The whole milk was pasteurized at 72°C using an ACG pasteurizer with a holding time of 17.31s and split into 3 portions. The first portion (about 120 kg) of the whole milk was allowed to gravity separate in a 120 liter cone bottom plastic gravity separation tank for 22 h at 4°C to obtain the gravity skim milk (ca 2.2% fat) and creams for use on day 2. The second portion (about 80 kg) of the pasteurized whole milk was separated using a De Laval cream separator **(**Model 619, Poughkeepsie, NY**)** at 50°C. The skim milk and cream were collected and recombined to obtain the same fat content of the whole milk, designated as recombined pasteurized whole milk. The third portion of the pasteurized whole milk (about 40 kg) was heated at 76°C for 7 min to stop gravity separation, to obtain high

temperature pasteurized whole milk that was designed to inactivate the gravity separation of properties of milk (Caplan et al., 2013).

For Replicate 2 and Replicate 3, a portion of the skim milk from the cream separator was saved and half the skim milk was heated at 76°C for 7 min. The skim milks were cooled to 4°C by manual stirring in a stainless steel milk can placed in an ice bath. The pasteurized skim milk (72°C for 17.31s) and high pasteurized skim milk (76°C for 7 min) were added to separate 20L cone-bottom gravity separation tanks. They were allowed to gravity separate at 4°C for 22 hours. A third replicate for the skim milk was performed on a separate date from a different batch of milk, using a tubular heat exchanger to cool the milks instead of cooling in milk cans in ice.

Nine clean one-liter glass gravity separation columns with a Teflon stopcock at the bottom (cat no. 03-789-5c, Fisher Scientific, Pittsburg, PA) and a Pasteur pipette bulb (cat no. 03-448-22, Fisher Scientific) on the bottom outlet with the stopcock closed and the top of the column capped with a rubber stopper were placed into a 4°C cooler prior to starting the experiment so that the columns would be cold before the milk addition. Five different treatments were performed on day 1 and 4 treatments were performed on day 2. On day 1, the five treatments were: 1) LTP whole milk, 2) LTP whole milk with added bacteria and spores, 3) recombined LTP whole milk with added bacteria and spores, 4) HTP whole milk with added bacteria and spores, and 5) HTP whole milk with added bacteria and spores and added colostrum. The target colostrum addition was 4.0 g/L of added Ig. All milks were cooled to 4°C before being added to the glass columns.

To ensure thorough mixing of the milk and any additions prior to pouring into the glass column, approximately 1,050 g of milk were poured into a 1.9 L plastic container and then any appropriate additions were added. The milk and additions were mixed thoroughly, about 50 g was poured into a 60 mL sterile plastic snap-top vial (cat no. CPP03CL, Capital Vial Inc., Amsterdam, NY) for analysis, and the rest was weighed and poured into the glass column and then the empty container was weighed again. The weight of the milk solution in each column

was recorded. The columns were closed and immediately placed in a 4°C cooler and the milk was allowed to gravity separate for 22 h.

Day 2. On day 2, the 5 glass columns filled on day 1 were drained starting from the bottom of the column in 5 fractions by weight: 0 to 5%, 5 to 90%, 90 to 96%, 96 to 98%, and 98 to 100%. The 5 to 90% fraction was collected by weight into a plastic 1.9 L container. The other 4 fractions were collected into sterile plastic snap-top vials (cat no. CPP02CL, Capital Vial Inc., Amsterdam, NY). The fractions collected were placed immediately into a 4°C cooler. The two 20L gravity separation tanks containing skim milk were drained from the bottom into 4 fractions by weight: 0 to 90%, 90 to 96%, 96 to 98%, and 98 to 100%. The fractions were collected into sanitized buckets, mixed thoroughly, and a samples collected in sterile plastic snap-top vials.

The 120 L cone-bottom plastic gravity separation tank was drained from the bottom into 3 fractions, 0 to 90%, 90 to 98%, and 98 to 100%. The 0 to 90% fraction, designated as gravity separated (GS) skim milk, was low in SC (~850 cells/mL). The 90 to 98% fraction was discarded. The 98 to 100% fraction was designated as cream high in SC (~5,500,000 cells/mL). The GS skim milk was separated using a DeLaval cream separator at 50°C. The skim collected, designated as centrifugal separated (CS) GS skim, was heated at 76°C for 7 mins to stop gravity separation. The cream from the cream separator was also collected and designated as cream low in SC (< 1000 cells/mL). The low SC and high SC creams were heated in glass (250 mL) Erlenmeyer flasks in a water bath at 76°C for 7 min to inactivate their gravity separation property.

Four different gravity separation treatments were started in glass columns on day 2: 1) HTP CS GS skim milk with HTP low SC cream, 2) HTP CS GS skim milk with HTP high SC cream, 3) HTP CS GS skim milk with HTP low SC cream and added colostrum, and 4) HTP CS GS skim milk with HTP high SC cream and added colostrum. The colostrum was added to achieve a target amount of 4.0 g/L of Ig. The same colostrum that was used on day 1 was used on day 2. The skim milk and creams used in the above 4 treatments were combined to achieve the target fat of the whole milk from day 1. All four treatments had an addition of bacteria and

spores. The same procedure was performed in adding the milks and additions to the glass columns as for day 1. All milks and creams were cooled to 4°C before being added to the glass columns. The four glass columns were allowed to gravity separate for 22 h at 4°C and the same 5 fractions were collected on day 3 as collected from the columns on day 2.

Analysis of Milk Fractions

The fat, SCC, standard plate count (SPC), and mesophilic spore count (MSC) were measured for the fractions collected from the various gravity separation treatments as well as on the initial samples collected. The fat content was determined (AOAC 2000: method 972.16) using a Fourier transform infrared dairy analyzer (LactoScope FTIR, Delta Instruments, Drachten, The Netherlands). The calibration of the infrared milk analyzer was done as described by Kaylegian et al. (2006a and b) using a set of modified milk calibration samples. The SCC was determined (AOAC 2000: method 17.13.01; 978.26) using fluorescence flow cytometry (SomaScope, Delta Instruments, Drachten De Boulder 68, The Netherlands). Milk samples outside of the range of the instrument calibration (i.e., > 1,000,000 SCC/mL) were diluted (w/w) using UF permeate. A dilution factor was used to calculate the SCC of the undiluted fraction. Samples high in fat were also diluted with UF permeate before SCC analysis to reduce their viscosity. Due to low volume of milk samples from the 96 to 98% and 98 to 100% fraction from the glass columns, those milk samples were diluted with UF permeate before both fat and SCC analysis to ensure enough volume for analysis.

Method #6.040 was used for SPC analysis (Laird et al., 2004). A modified method #8.090 (Frank and Yousef, 2004) was used for MSC analysis. Due to low volume of the top column fractions, 5 mL of the milk was heated to 80°C for 12 minutes instead of 200 mL as stated in the method. For both SPC and MSC analysis, appropriate dilutions of the samples were made prior to plating to ensure plate counts were in the countable range (between 25 and 250 cells/mL).

RESULTS AND DISCUSSION

Composition of Starting Whole Milks

The initial fat, SC, bacteria, and spores for the 9 treatments are shown in Table 3.1. No difference in initial percentage of fat ($P > 0.05$) was detected among treatments except for the recombined LTP whole with added bacteria and spores which had a higher percentage of fat ($P < 0.05$). This was due to an error in recombining the skim and cream in Replicate 1. There was no difference detected ($P > 0.05$) in the initial SCC in the LTP whole milk, LTP whole milk with added bacteria and spores, HTP whole milk with added bacteria and spores, and HTP whole milk with added bacteria, spores, and colostrum. The recombined LTP whole milk with added bacteria and spores had a lower initial SCC count ($P < 0.05$) due to the removal of SC during the centrifugal cream separation process. The HTP CS GS skim milk with HTP high SC cream with added bacteria and spores and HTP CS GS skim milk with HTP high SC cream with added bacteria, spores, and colostrum had the highest initial SCC ($P < 0.05$) due to the addition of high SC cream. As expected, the HTP CS GS skim milk with HTP low SC cream with added bacteria and spores and HTP CS GS skim milk with HTP low SC cream with added bacteria, spores, and colostrum had the lowest initial SCC ($P < 0.05$) due to the gravity separation removal of SC in the HTP CS GS skim milk and the low SC in the cream.

Table 3.1. Mean (n=3) initial values for fat, somatic cells (SC), bacteria, and spores in each column of low temperature (72°C for 17.3s) pasteurized (LTP) and high temperature (76°C for 7 min) pasteurized (HTP) whole milk.

Treatment	% Fat	SC (SCC[1]/mL)	Bacteria (SPC[2]/mL)	Spores (MSC[3]/mL)
LTP whole	3.62[a]	285,000[b]	8[c]	7[b]
LTP whole + bacteria + spores	3.61[a]	296,333[b]	1,773,333[b]	46,000[a]
Recombined LTP whole + bacteria + spores	3.90[b]	106,500[c]	2,088,333[a]	48,333[a]
HTP whole + bacteria + spores	3.64[a]	293,833[b]	2,095,000[a]	45,833[a]
HTP whole + bacteria + spores + colostrum	3.56[a]	306,167[b]	2,083,333[a]	47,667[a]
HTP CS GS[4] skim with HTP low SC cream + bacteria + spores	3.61[a]	1,000[d]	2,236,667[a]	49,500[a]
HTP CS GS skim with HTP high SC cream + bacteria + spores	3.63[a]	560,000[a]	2,045,000[a,b]	50,667[a]
HTP CS GS skim with HTP low SC cream + bacteria + spores + colostrum	3.54[a]	20,833[d]	2,111,667[a]	42,333[a]
HTP CS GS skim with HTP high SC cream + bacteria + spores+ colostrum	3.58[a]	598,833[a]	2,085,000[a]	49,833[a]
R-squared	0.693	0.972	0.960	0.876
S.E.	0.065	25,865	101,597	5,875

[a-e]Means in the same column not sharing a common superscript are different ($P < 0.05$)
[1]SCC = Somatic cell count
[2]SPC = Standard plate count
[3]MSC = Mesophilic spore count
[4] CS GS = Centrifugally separated, gravity separated skim milk

The LTP whole milk had a much lower initial bacteria count ($P < 0.05$), as this was the only treatment that did not have the addition of bacteria. The LTP whole milk with added bacteria and spores had a different initial bacteria count ($P < 0.05$) from all the treatments except HTP CS GS skim milk with HTP high SC cream with added bacteria and spores. There was no difference detected ($P > 0.05$) among the initial bacteria count for the other treatments that had the addition of bacteria. The lower initial bacteria count in the LTP whole milk with added bacteria and spores most likely was due to random variation in the bacteria solution used. As expected, the LTP whole milk had a much lower initial spore count ($P < 0.05$) than the other treatments due to the addition of spores in all other treatments. There was no difference detected in the initial spore count ($P > 0.05$) between the treatments that had added spores.

Gravity Separation of Fat, SC, Bacteria, and Spores in Whole Milks

Gravity Separation of Fat. If there was no gravity separation of fat, the expected percentage of fat present in each fraction would be equal to the percentage mass of that column (i.e. 5% of the fat present in the 0 to 5% fraction, 85% in the 5 to 90% fraction, etc.). As expected based on the results of Caplan et al. (2013), gravity separation of fat occurred in the LTP whole milk and LTP whole milk with added bacteria and spores (i.e., about 50% of the fat in the 10% of the column instead of 10% of the fat) and no difference in the percentage of fat present in the different fractions of these 2 treatments (Table 3.2) was detected ($P > 0.05$). While recombined LTP whole milk with added bacteria and spores showed gravity separation of fat, the amount of fat in the top 10% was lower ($P < 0.05$) than LTP whole milk and LTP whole with added bacteria and spores. The process of CS separating skim milk and cream and recombining of cream and skim milk reduced ($P < 0.05$) the gravity separation of fat, although it did not stop it.

Four treatments consistently had very little or no gravity separation of fat: HTP whole milk with added bacteria and spores, HTP CS GS skim milk with HTP low SC cream with added bacteria and spores, HTP CS GS skim milk with HTP high SC cream with added bacteria and spores, and HTP CS GS skim milk with HTP low SC cream with added bacteria, spores, and

colostrum. No difference ($P > 0.05$) in the percentage of fat present in the 5 to 90%, 90 to 96%, 96 to 98% and the top 10% for these 4 treatments was detected. The results from these 4 treatments indicate that: (1) HTP of whole milk stops gravity separation of fat and is consistent with the results of Caplan et al. 2013, (2) the gravity separation of fat in HTP whole milk is not restored by addition of SC, and (3) that addition of unheated colostrum when the presence of SC was low (i.e., ~21,000 cell/mL) did not restore gravity separation of fat.

For the treatment of HTP whole milk with added bacteria, spores, and colostrum, the addition of colostrum did partially restore the gravity separation of fat with the percentage of the total fat in the upper 10% being lower ($P < 0.05$) than the LTP whole milk (44.9 vs 51.8%, respectively), but still much more than 10% of that fat if no gravity separation occurred. The final treatment, HTP CS GS skim milk with HTP high SC cream with added bacteria, spores, and colostrum had a higher ($P < 0.05$) percentage of the fat in the top 10% (i.e., more gravity separation of fat) than all other treatments. When both SC and immunoglobulins from colostrum were added to HTP CS GS skim milk, the gravity separation properties of the milk was restored to a level $\geq$ to the LTP whole milk. The addition of both SC and Ig restored the gravity separation of fat of HTP milk to achieve a higher level of gravity separation than the original LTP whole milk.

Euber and Brunner (1984) identified IgM as the likely Ig involved in the gravity separation of fat. When IgM was added to milk that had high heat treatment, it restored the gravity separation of fat (Euber and Brunner, 1984). This is consistent with our observations the addition of colostrum restored the gravity separation of fat.

Table 3.2. Mean (n = 3) percentage of total fat in the 5 fractions in each column of low temperature (72°C for 17.3 s) pasteurized (LTP) and high temperature (76°C for 7 min) pasteurized (HTP) whole milk after gravity separation for 23 h at 4°C.

Treatment	0-5%	5-90%	90-96%	96-98%	98-100%	Top 10%
LTP whole	$1.6^{c,d}$	46.6^{d}	24.1^{a}	12.6^{b}	15.0^{a}	51.8^{b}
LTP whole + bacteria + spores	$1.6^{c,d}$	46.0^{d}	24.0^{a}	13.1^{b}	15.2^{a}	52.3^{b}
Recombined LTP whole + bacteria + spores	1.5^{d}	68.3^{b}	10.2^{c}	9.3^{c}	10.7^{b}	30.2^{d}
HTP whole + bacteria + spores	1.8^{c}	85.1^{a}	6.1^{d}	2.0^{d}	5.0^{c}	13.2^{e}
HTP whole + bacteria + spores + colostrum	1.5^{d}	53.5^{c}	16.9^{b}	13.3^{b}	14.8^{a}	44.9^{c}
HTP CS GS[1] skim with HTP low SC[2] cream + bacteria + spores	3.1^{a}	86.4^{a}	6.0^{d}	1.9^{d}	2.6^{d}	10.5^{e}
HTP CS GS skim with HTP high SC cream + bacteria + spores	1.4^{d}	85.1^{a}	6.0^{d}	2.3^{d}	5.1^{c}	13.5^{e}
HTP CS GS skim with HTP low SC cream + bacteria + spores + colostrum	2.8^{b}	83.7^{a}	6.0^{d}	1.9^{d}	5.7^{c}	13.6^{e}
HTP CS GS skim with HTP high SC cream + bacteria + spores+ colostrum	0.9^{e}	40.8^{e}	24.4^{a}	17.1^{a}	17.0^{a}	58.4^{a}
R-squared	0.974	0.992	0.958	0.986	0.964	0.991
S.E.	0.079	1.271	1.276	0.514	0.762	1.326

$^{a-e}$Means in the same column not sharing a common superscript are different ($P < 0.05$)

[1] CS GS = Centrifugally separated, gravity separated skim milk

[2] SC = Somatic cell

Gravity Separation of SC. If there was no gravity separation of SC, the expected percentage of SC present in each fraction would be equal to the percentage mass of that column (i.e. 5% of the SC present in the 0 to 5% fraction, 85% in the 5 to 90% fraction, etc.). Two treatments, (1) HTP CS GS skim milk with HTP low SC cream with added bacteria and spores and (2) HTP CS GS skim milk with HTP low SC cream with added bacteria, spores, and colostrum, were not included in the data for the gravity separation of SC (Table 3.3) due to the very low total SC in the milk prior to gravity separation (Table 3.1). As expected, LTP whole milk and LTP whole milk with added bacteria and spores both showed gravity separation of SC in all the fractions (Table 3), with no differences ($P > 0.05$) in any of the fractions between the 2 treatments. This is consistent with the results from Caplan et al. (2013). No difference was detected ($P > 0.05$) in mean percentage of SC of the recombined LTP whole milk with added bacteria and spores from the LTP whole milk for the gravity separation of SC in any of the fractions, indicating that the process of CS separating skim milk and cream recombining of cream and skim had little, if any, effect on the gravity separation of SC.

There were 2 treatments that consistently did not have gravity separation of SC: (1) HTP whole milk with added bacteria and spores and (2) HTP CS GS skim milk with HTP high SC cream with added bacteria and spores. No difference was detected ($P > 0.05$) in the percentage of SC present for these 2 treatments in all the fractions except the 0 to 5% layer. Both these treatments were different ($P < 0.05$) than the LTP whole in all the fractions. We conclude that (1) HTP of whole milk stops gravity separation of SC and is consistent with the results of Caplan et al. 2013 and (2) the gravity separation of SC in HTP whole milk was not restored by addition of SC.

The SC in HTP whole milk with added bacteria, spores, and colostrum gravity separated (Table 3.3) (i.e., 95.8% of the SC in the top 10% of the column). The addition of colostrum did partially restore the gravity separation of SC. The HTP whole milk with added bacteria, spores, and colostrum contained a lower percentage of SC present for the 98 to 100% and top 10% fraction (i.e., 95.8 vs 99.7%, respectively) and a higher percentage present for the 5 to 90%

fraction than the LTP whole milk ($P < 0.05$). The combination of addition of both SC and colostrum to HTP CS GS milk restored the gravity separation of SC, as seen from the result (Table 3.3) that no difference ($P > 0.05$) in the proportion of total SC in the top 10% of the HTP CS GS skim with HTP high SC cream with added bacteria, spores, and colostrum and the LTP whole milk was detected.

Table 3.3. Mean (n = 3) percentage of total somatic cells (SC) in the 5 fractions in each column of low temperature (72°C for 17.3 s) pasteurized (LTP) and high temperature (76°C for 7 min) pasteurized (HTP) whole milk after gravity separation for 23 h at 4°C.

Treatment	0-5%	5-90%	90-96%	96-98%	98-100%	Top 10%
LTP whole	0.2^{c}	0.08^{c}	$17.0^{b,c}$	30.9^{a}	51.8^{a}	99.7^{a}
LTP whole + bacteria + spores	0.2^{c}	0.2^{c}	$17.4^{b,c}$	31.2^{a}	51.1^{a}	99.7^{a}
Recombined LTP whole + bacteria + spores	0.08^{c}	0.0^{c}	$9.7^{c,d}$	33.8^{a}	56.3^{a}	99.9^{a}
HTP whole + bacteria + spores	6.3^{a}	83.2^{a}	5.4^{d}	1.8^{b}	3.3^{c}	10.5^{c}
HTP whole + bacteria + spores + colostrum	0.5^{c}	3.7^{b}	$21.9^{a,b}$	31.2^{a}	42.7^{b}	95.8^{b}
HTP CS GS[1] skim with HTP low SC cream + bacteria + spores	-	-	-	-	-	-
HTP CS GS skim with HTP high SC cream + bacteria + spores	3.9^{b}	84.1^{a}	5.7^{d}	2.0^{b}	4.3^{c}	12.0^{c}
HTP CS GS skim with HTP low SC cream + bacteria + spores + colostrum	-	-	-	-	-	-
HTP CS GS skim with HTP high SC cream + bacteria + spores+ colostrum	0.3^{c}	1.0^{c}	25.3^{a}	32.0^{a}	41.5^{b}	98.8^{a}
R-squared	0.985	1.000	0.830	0.989	0.971	1.000
S.E.	0.215	0.561	2.604	1.107	2.751	0.574

[a-d]Means in the same column not sharing a common superscript are different ($P < 0.05$)
[1] CS GS = Centrifugally separated, gravity separated skim milk

Gravity Separation of Bacteria. If there was no gravity separation of bacteria, the expected percentage of bacteria present in each fraction would be equal to the percentage mass of that column (i.e. 5% of the bacteria present in the 0 to 5% fraction, 85% in the 5 to 90% fraction, etc.). The bacteria in both LTP whole milk and LTP whole milk with added bacteria and spores gravity separated (Table 3.4). Thus, both the bacteria present in the LTP milk and the bacteria that we added to the milk gravity separated. Anderson (1909) observed that gravity separation concentrated the bacteria in the cream layer and that 10 to 500 times as many bacteria were in the cream layer as compared to the starting milk. Dellaglio et al. (1969) found that bacteria was concentrated in the cream layer (which was defined as the top 16%) after gravity separation.

Three treatments showed little or no gravity separation of bacteria: (1) HTP whole milk with added bacteria and spores, (2) HTP CS GS skim milk with HTP low SC cream with added bacteria and spores, and (3) HTP CS GS skim milk with HTP high SC cream with added bacteria and spores. We hypothesize that that this was due to the heat inactivation of the Ig that was originally present in the milk. No differences were detected ($P > 0.05$) in any of the fractions for these three treatments. The HTP treatment stopped the gravity separation of bacteria and the addition of SC did not restore the gravity separation of bacteria (Table 3.4).

There was also one treatment that had partial gravity separation of bacteria: HTP CS GS skim milk with HTP low SC cream with added bacteria, spores, and colostrum had more ($P < 0.05$) bacteria present in the top 10% fraction than the 3 treatments where the bacteria did not gravity separate (Table 3.4). The slight elevation of SC from the colostrum, as shown in Table 3.1, could be causing the partial gravity separation of bacteria.

No difference ($P > 0.05$) in the percentage of total bacteria in the recombined LTP whole milk with added bacteria and spores and from the LTP whole milk with added bacteria and spores was detected in the various fractions, except for the 90 to 96% fraction. Overall, little or no effect of the process of CS separating skim milk and cream recombining of cream and skim milk on the gravity separation of bacteria was detected. The bacteria in the two treatments: (1)

HTP whole milk with added bacteria, spores, and colostrum and (2) HTP CS GS skim milk with HTP high SC cream with added bacteria, spores, and colostrum gravity separated (Table 4) but the proportion of the total bacteria that rose to the top 10% layer was lower ($P < 0.05$) than LTP whole (95.4%). The addition of colostrum, presumably the Ig in the colostrum added in these treatments, did restore gravity separation of bacteria, but the proportion of the total bacteria in the top 10% was slightly lower (Table 3.4). This is consistent with observations by Stadhouders and Hup (1970) that showed that antibodies caused to bacteria to agglutinate in milk and become attached to the fat globules, suggesting a similar agglutinating process for fat and bacteria.

Table 3.4. Mean (n = 3) percentage of total bacteria in the 5 fractions in each column of low temperature (72°C for 17.3 s) pasteurized (LTP) and high temperature (76°C for 7 min) pasteurized (HTP) whole milk after gravity separation for 23 h at 4°C

Treatment	0-5%	5-90%	90-96%	96-98%	98-100%	Top 10%
LTP whole	0.2^{d}	4.4^{e}	15.1^{c}	29.3^{a}	50.9^{a}	95.4^{a}
LTP whole + bacteria + spores	$0.4^{c,d}$	$9.9^{d,e}$	33.5^{b}	25.1^{a}	$31.1^{b,c}$	$89.7^{a,b}$
Recombined LTP whole + bacteria + spores	$0.4^{c,d}$	$13.9^{c,d}$	20.1^{c}	29.6^{a}	35.9^{b}	$85.7^{b,c}$
HTP whole + bacteria + spores	5.0^{a}	85.3^{a}	5.9^{d}	1.9^{b}	2.0^{e}	9.7^{e}
HTP whole + bacteria + spores + colostrum	0.9^{c}	19.5^{c}	31.0^{b}	24.3^{a}	$24.3^{b,d}$	79.6^{c}
HTP CS GS[1] skim with HTP low SC[2] cream + bacteria + spores	4.9^{a}	85.9^{a}	5.6^{d}	2.0^{b}	1.7^{e}	9.3^{e}
HTP CS GS skim with HTP high SC cream + bacteria + spores	4.8^{a}	86.1^{a}	5.6^{d}	1.7^{b}	1.8^{e}	9.1^{e}
HTP CS GS skim with HTP low SC cream + bacteria + spores + colostrum	2.7^{b}	75.7^{b}	5.4^{d}	1.8^{b}	$14.3^{d,e}$	21.6^{d}
HTP CS GS skim with HTP high SC cream + bacteria + spores+ colostrum	$0.8^{c,d}$	$16.0^{c,d}$	40.9^{a}	23.0^{a}	$19.3^{c,d}$	$83.3^{b,c}$
R-squared	0.981	0.994	0.947	0.874	0.889	0.994
S.E.	0.206	2.090	2.351	3.554	4.409	2.136

[a-e]Means in the same column not sharing a common superscript are different ($P < 0.05$)

[1] CS GS = Centrifugally separated, gravity separated skim milk

[2] SC = Somatic cell

Gravity Separation of Spores. If there was no gravity separation of spores, the expected percentage of spores present in each fraction would be equal to the percentage mass of that column (i.e. 5% of the spores present in the 0 to 5% fraction, 85% in the 5 to 90% fraction, etc.). Both LTP whole milk and LTP whole milk with added bacteria and spores had the gravity separation of spores with > 90% of the spores present in the top 10% layer (Table 3.5). Both Rossi (1964) and Dellaglio et al. (1969) showed evidence that spores rise to the top during the gravity separation process.

No gravity separation was detected ($P > 0.05$) in three treatments: (1) HTP whole with added bacteria and spores, (2) HTP CS GS skim milk with HTP low SC cream with added bacteria and spores, and (3) HTP CS GS skim milk the HTP high SC cream with added bacteria and spores (Table 3.5). The HTP CS GS skim milk with HTP low SC cream with added bacteria, spores, and colostrum showed a partial gravity separation and we speculate that the slight addition of SC from the colostrum could be causing the partial gravity separation of spores (i.e., 23.3% of the spores in the top 10%). The results of these 4 treatments show that: (1) HTP treatment stops the gravity separation of spores, (2) the addition of just SC does not restore gravity separation of spores and (3) the addition of colostrum Ig at low SC does not completely restore the gravity separation of spores.

The spores in the recombined LTP whole with added bacteria and spores gravity separated with about 87.9% of the spores present in the top 10% (Table 3.5) which was lower ($P < 0.05$) than the LTP whole milk (96.9%) The process of CS separating skim milk and cream recombining of cream and skim milk caused a small reduction in gravity separation of spores. No difference ($P > 0.05$) in spores in the top 10% among the HTP whole with added bacteria, spores, and colostrum and both the recombined LTP whole with added bacteria and spores and the LTP whole with added bacteria and spores was detected. The addition of colostrum to HTP whole milk (containing SC) restored the gravity separation of spores.

No difference in gravity separation of spores in the top 10% layer in the HTP CS GS skim with HTP high SC cream with added bacteria, spores, and colostrum and the LTP whole

with added bacteria and spores was detected. Therefore, addition of Ig alone was not sufficient to cause spores to rise to the top in gravity separation. The addition of both SC and Ig from colostrum to the HTP milk restored the gravity separation of spores and demonstrated that both SC and Ig are important for the rising of spores in gravity separation. We hypothesize that gas within the SC provided the buoyancy that causes spores, SC, fat and bacteria to rise during gravity separation in aggregates formed by the interaction with immunoglobulins. This behavior is consistent with the fact that practical experience has shown that standardization of milk fat content for production of Grana Padano and Parmigiano Reggiano cheese production produces cheese with fewer gas defects than when the milks for production of the cheeses are standardized by centrifugal cream separation.

Table 3.5. Mean (n = 3) percentage of total spores in the 5 fractions in each column of low temperature (72°C for 17.3s) pasteurized (LTP) and high temperature (76°C for 7 min) pasteurized (HTP) whole milk after gravity separation for 23 h at 4°C

Treatment	0-5%	5-90%	90-96%	96-98%	98-100%	Top 10%
LTP whole	1.2^{c}	1.9^{d}	36.9^{a,b}	25.3^{b}	34.7^{a}	96.9^{a}
LTP whole + bacteria + spores	0.5^{c}	8.2^{c,d}	40.9^{a}	25.2^{b}	25.2^{b,c}	91.3^{a,b}
Recombined LTP whole + bacteria + spores	0.5^{c}	13.5^{c}	20.7^{d}	33.6^{a}	31.7^{a,b}	86.0^{b}
HTP whole + bacteria + spores	8.2^{a}	79.5^{a,b}	8.3^{e}	2.6^{c}	1.3^{d}	12.3^{d}
HTP whole + bacteria + spores + colostrum	0.5^{c}	11.7^{c}	28.9^{c}	30.5^{a,b}	28.5^{a,b}	87.9^{b}
HTP CS GS[1] skim with HTP low SC[2] cream + bacteria + spores	6.0^{b}	85.4^{a}	5.8^{e}	1.6^{c}	1.2^{d}	8.6^{d}
HTP CS GS skim with HTP high SC cream + bacteria + spores	6.2^{a,b}	84.1^{a}	6.0^{e}	2.0^{c}	1.7^{d}	9.7^{d}
HTP CS GS skim with HTP low SC cream + bacteria + spores + colostrum	2.5^{c}	74.3^{b}	4.0^{e}	1.5^{c}	17.8^{c}	23.3^{c}
HTP CS GS skim with HTP high SC cream + bacteria + spores+ colostrum	0.8^{c}	15.0^{c}	32.0^{b,c}	25.9^{b}	26.3^{a,b,c}	84.1^{b}
R-squared	0.905	0.988	0.938	0.934	0.912	0.990
S.E.	0.700	2.960	2.695	2.654	3.077	2.887

[a-e]Means in the same column not sharing a common superscript are different ($P < 0.05$)
[1] CS GS = Centrifugally separated, gravity separated skim milk
[2] SC = Somatic cell

Summary of the Gravity Separation in Whole Milks. Gravity separation of fat, SC, bacteria, and spores was observed in LTP whole milk, LTP whole with added bacteria and spores, and recombined LTP whole milk with added bacteria and spores (Tables 2, 3, 4, and 5). Gravity separation was not observed in HTP whole milk with added bacteria and spores, indicating the HTP treatment stopped the gravity separation of all components due to thermal inactivation of Ig. Colostrum restored the gravity separation of all 4 components in HTP whole milk containing a normal level of SC, as shown in the HTP whole milk with added bacteria, spores, and colostrum. If both SC and Ig were not present, there was little to no gravity separation observed as seen in the HTP CS GS skim milk with HTP low SC cream with added bacteria and spores, HTP CS GS skim milk with HTP high SC cream with added bacteria and spores, and HTP CS GS skim milk with HTP low SC cream with added bacteria, spores, and colostrum (Tables 2, 3, 4, and 5). The addition of SC and Ig restored the gravity separation of fat, SC, bacteria, and spores in whole milk, shown in the HTP CS GS skim milk with HTP high SC cream with added bacteria, spores and colostrum.

The interaction between SC and Ig was necessary to cause aggregates of fat, SC, bacteria, and spores to rise during gravity separation. Frenyo et al. (1986) observed that IgM and IgA, but not IgG1 and IgG2, associated with leukocytes, i.e. somatic cells, in milk. The SC may provide the buoyancy required for the aggregates to rise to the top due to gas within the SC. White blood cells (neutrophils) are known to produce oxygen, as they kill phagocytized bacterial cells and produce hydrogen peroxide that is degraded by catalase to form oxygen within the cell (Parham, 2009). This process is called respiratory burst.

Gravity Separation of SC, Bacteria, and Spores in Skim Milks

The initial values for fat, SC, bacteria, and spores in the LTP and HTP skim milks are shown in Table 3.6. There was no difference detected ($P > 0.05$) between the two treatments for starting fat and SC. There was a difference detected ($P < 0.05$) for the starting bacteria count between the LTP and HTP skim milk. The difference in starting bacteria may have been due to post pasteurization contamination. There was a difference detected ($P < 0.05$) for the starting

spore count between the LTP and HTP skim milk. Some of the differences detected for spores counts may have been due to limitations in detecting the spores in the milk due to such low counts.

Table 3.6. Mean (n = 3) initial values for fat, somatic cells (SC), bacteria, and spores in the treatments of low temperature (72°C for 17.3s) pasteurized (LTP) and high temperature (76°C for 7 min) pasteurized (HTP) skim milk.

Treatment	% Fat	SC (SCC[1]/mL)	Bacteria (SPC[2]/mL)	Spores (MSC[3]/mL)
LTP skim	0.09[a]	114,000[a]	1,038[a]	9[b]
HTP Skim	0.09 [a]	121,000[a]	354[b]	16[a]
R-squared	0.601	0.882	0.999	0.984
S.E.	0.001	2,884	23	0.97

[a-b]Means in the same column not sharing a common superscript are different ($P < 0.05$)
[1] SCC = Somatic cell count
[2] SPC = Standard plate count
[3] MSC = Mesophilic spore count

The percentage of SC, bacteria, and spores present in the 0 to 96% and 96 to 100% fractions for the LTP skim milk and HTP skim milk are shown in Table 3.7. For the percentage of SC, bacteria, and spores in both fractions, there was a difference ($P < 0.05$) between the LTP skim milk and HTP skim milk. The LTP skim milk had a lower percentage ($P < 0.05$) of each component in the 0 to 96% fraction and higher percentage ($P < 0.05$) of each component in the 96 to 100% fraction. If there was no gravity separation present, the expected percentage of each component would be 96% present in the 0 to 96% layer and 4% present in the 96 to 100% layer. For the LTP skim, there was much more than 4% present in the 96 to 100% layer, showing that SC, bacteria, and spores gravity separated. For the HTP skim milk, there was no gravity separation present for SC, bacteria, and spores. Two important conclusions: (1) SC, bacteria, and spores gravity separate in LTP skim milk (i.e., gravity separation is not dependent on the presence of fat) and (2) HTP treatment stops the gravity separation of SC, bacteria, and spores in skim milk just like it does in whole milk.

Table 3.7. Mean (n = 3) percentage of somatic cells (SC), bacteria, and spores present in the 0 to 96% and 96 to 100% fractions in low temperature (72°C for 17.3s) pasteurized (LTP) and high temperature (76°C for 7 min) pasteurized (HTP) skim after gravity separation for 23 h at 4°C.

Treatment	% SC 0 – 96%	% SC 96 – 100%	% Bacteria 0 – 96%	% Bacteria 96 – 100%	% Spore 0 – 96%	% Spore 96 – 100%
LTP Skim	44.8[b]	55.2[a]	70.2[b]	29.8[a]	53.8[b]	46.2[a]
HTP Skim	93.9[a]	6.1[b]	92.4[a]	7.6[b]	94.0[a]	6.0[b]
R-squared	0.9997	0.9997	0.993	0.993	0.987	0.987
S.E.	0.252	0.252	0.720	0.720	1.457	1.467

[a-b]Means in the same column not sharing a common superscript are different ($P < 0.05$)

CONCLUSIONS

The presence of both SC and Ig were necessary for normal gravity separation (i.e., rising to the top) of fat, bacteria, and spores in whole milk. The presence of Ig alone without somatic cells was not sufficient to cause the rising of bacteria, fat and spores to the top without SC. The interaction between SC and Ig was necessary to cause aggregates of fat, SC, bacteria, and spores to rise during gravity separation. The SC may provide the buoyancy required for the aggregates to rise to the top due to gas within the SC. More research is needed to understand the mechanism of the gravity separation process.

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CHAPTER FOUR

Conclusions and Future Research Needs

CONCLUSIONS

In the first set of experiments we found that heat treatment of skim milk (76°C for 7 min) was sufficient to stop the gravity separation of somatic cells. Colostrum (as a source of Ig) added to heated (76°C for 7 min) skim milk restored the gravity separation of somatic cells. We conclude that Ig are at least one of the factors necessary for the gravity separation of SC, bacteria, and bacterial spores in milk.

In the second set of experiments we found that the presence of both somatic cells and Ig were necessary for normal gravity separation (i.e., rising to the top) of fat, bacteria, and spores in whole milk. The presence of Ig alone without somatic cells was not sufficient to cause the rising of fat, bacteria and spores to the top without SC. The interaction between SC and Ig was necessary to cause aggregates of fat, SC, bacteria, and spores to rise during gravity separation. The SC may provide the buoyancy required for the aggregates to rise to the top due to gas within the SC.

Possible Applications for Gravity Separation

Even though gravity separation is considered an old technique for removing fat from milk, there are still some possible applications for gravity separation today. Small artisan dairy product manufacturers can standardize their milk with different fat percentages without the need to purchase an expensive cream separator. The gravity separation process also allows some of the bacteria and spores to be removed from the milk, increasing the safety and quality of the milk products.

The fact that bacteria and spores gravity separate also leads to some potential applications. In dairy products, like fluid milk, milk powder and Swiss cheese, spores can cause defects in the final product. Removing spores from fluid milk increases the shelf life (Andersson et al., 1995). For Swiss cheese, *Clostridium tyrobutyricum* can cause butyric acid fermentation which negatively affects flavor and *Clostridium sporogenes* can cause intense proteolysis. In

order to avoid these problems, the spores either need to be eliminated through processing or have their germination suppressed prior to cheese making or feed cows a silage-free diet (Bachmann et al., 2011). Spores in milk powders can cause flavor defects (Burgess et al., 2010). Spores are very resilient and difficult to eliminate due to their high resistance to thermal processing (Burgess et al., 2010). Gravity separation offers an easy way to remove a portion of the spores present in milk prior to production of some dairy products.

One of the drawbacks of gravity separation is the time required. By understanding the mechanism of what is required for the gravity separation of bacteria and spores, it may be possible to develop a continuous flow process to remove bacteria and spores from fluid milk. Ig could coat a filter that traps bacteria and spores as milk flows through it. A type of regeneration cycle could be used to remove the bacteria and spores from the filter without damaging to Ig so the filter could be reused. This would take advantage of the benefits of gravity separation while eliminating the longer time required needed for gravity separation take place.

FUTURE RESEARCH NEEDS

In order to fully understand the gravity separation process, more research needs to be performed analyzing the types of bacteria and spores that gravity separate. Bacteria and spores can differ from one another in their surface chemistry. By analyzing which type(s) of bacteria and spores gravity separate and which don't, it will lead to better understanding of the agglutination process and it specificity. There is also evidence that vaccination can change the Ig composition of the milk, based of analysis of J5 vaccination towards *Escherichia coli* O111:B4 and vaccination towards *Staphylococcus aureus* (Tempelmans Plat-Sinnige et al., 2009, Wilson et al., 2009). Vaccination of cows in the future may be used to target the gravity separation of specific bacteria and spores.

It is still not clear which Ig (IgA, IgG, or IgM) plays a role in the gravity separation of bacteria and spores. Future work involves using ELISA to analyze the Ig content in the different milk fractions after gravity separation. This technique will allow us to see which specific Ig rises

to the top after gravity separation, and is therefore likely involved in the gravity separation process.

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