Analysis of Alternative Donor Breast Milk Pasteurization Techniques and Their Impact on Bile Salt-Stimulated Lipase Activity

by
Ashley Faye Victor

A THESIS

submitted to

Oregon State University

Honors College

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degree of

Honors Baccalaureate of Science in Biology
(Honors Scholar)

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While breast milk is considered the best nourishment for newborns, they have a lowered ability to digest the fat rich liquid due to decreased lipase secretion for the first few months after birth. To offset this, breast milk itself contains Bile Salt-Stimulated Lipase (BSSL) which significantly heightens fat digestion ability. Pre-term infants (<37 weeks gestation age) are often fed donor breast milk, which is treated through Holder pasteurization by nonprofit milk banks to ensure microbiological safety. Due to the fact that Holder inactivates up to 95% of BSSL, two alternative techniques also proven to eliminate harmful breast milk pathogens were assessed for their ability to retain BSSL: high pressure processing (HPP) and gamma cell irradiation (IR). After treatment of breast milk samples with either Holder (62.5°C, 30 min), HPP (550 MPa, 5 mins), or IR (2.5 Mrads), BSSL activity was determined using a spectrometric assay and compared to raw untreated milk. IR treatment was shown to retain 32% of BSSL activity while HPP was shown to retain 84%, suggesting HPP could be an effective alternative to Holder pasteurization.

Abstract approved:_____

David Dallas

Key Words: Lipase, Breast Milk, High Pressure Processing, Gamma Cell Irradiation

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Ashley Faye Victor, Author

Introduction

Though breast milk is the best available nourishment for the newborn term infant, they have lowered fat digestion capacity for the first few months post birth. The cause of this decreased capacity is hypothesized to be due to immature pancreatic development leading to secretions of low amounts of lipase.[1] At birth, one study found lipase concentrations for full term infants to be at 143.9 IU, which increased to 1464 IU once the infant was older than 9 months.[2] Preterm infants (<37 weeks gestation age) have even less ability to digest fat[3]: in these infants, as much as 20-30% of dietary fat in breast milk is not digested and absorbed.[4] This is supported by the fact that preterm lipase concentration was found to be 77.4 IU at birth, half that of the already lowered full-term concentration.[2] Impaired fat digestion is problematic because its digestion and absorption are essential to weight gain and brain development.

Breast milk contains bile salt-stimulated lipase (BSSL) which activates when milk reaches the infant's intestine and combines with bile salts and is a major contributor to the infant's ability to digest fat.[4] In comparing preterm infants fed a mixture of formula and human milk or exclusively formula, infants fed human milk digested up to 7.2% more dietary fat.[5] As mothers of premature infants very often cannot produce an adequate supply of breast milk, these infants are often fed donor milk that is processed and stored by milk banks. Unfortunately, this important enzyme is completely inactivated by holder pasteurization (62.5°C, 30 min), the bacterial and viral reduction technique currently used by all non-profit milk banks.[6] Pasteurization-induced lipase degradation reduces overall fat absorption capacity by 17–30% and reduces weight gain and linear growth in preterm infants.[1, 6, 7] Attempts to supplement pasteurized donor milk with recombinant bile salt-stimulated lipase failed to improve growth and showed increased gastrointestinal intolerance.[8] Holder pasteurization, or Low Temperature Long

Time (LTLT), remains the standard for microbiological safety because of its consistent ability to destroy bacteria routinely found in donor breast milk.[9] Exposure to pathogenic bacteria like group B streptococci, *Staphylococcus aureus*, *Salmonella* sp, *Klebsiella pneumoniae*, *Listeria monocytogenes*, and certain *Escherichia coli* strains is of concern because of the role they can play in neonatal sepsis, meningitis, necrotizing enterocolitis, and diarrheal disease, particularly for preterm and immunocompromised infants.[10,11] The FDA stipulates that pasteurization must meet a 5-log pathogenic microorganism reduction standard for the most common contaminants of the product, indicating that 99.99% of microorganisms will be destroyed.[12] Therefore, developing an alternative approach that reaches this standard while maintaining breast milk's native enzymes is a preferable strategy for improving premature infant growth.

High pressure processing (HPP), or high hydrostatic pressure, is a novel nonthermal food safety technology that is just beginning to be explored for the treatment of donor breast milk. Pressure units are designed to withstand between 100 and 800 MPa and can be set to maintain temperatures from below 0˚C to above 100˚C depending on needs. HPP acts instantaneously and uniformly on the entire sample regardless of samples size and composition.[13] Samples are vacuum-sealed in flexible and water-resistant packaging, submerged in water, and subjected to a predetermined pressure for a certain time.[14] HPP treatment of breast milk at 400 MPa for 4 min allows an 8-log reduction of S*treptococcus agalactiae* and 2 min for an 8-log reduction of *Listeria monocytogenes* with no destruction of milk secretory immunoglobulin A and lipoprotein lipase.[15,16,17] Unlike LTLT, there is not yet an agreed upon standard of the parameters for HPP treatment due to the fact that three variables can be adjusted: time, pressure, and temperature. Although the range of treatment options is large, microbiological safety has been shown to be achieved with pressures between 400 and 600 MPa for up to 5 minutes.[18] The effect of this high

pressure processing on bile salt-stimulated lipase remains unexamined but is expected to be minimal.

Gamma-cell irradiation (IR) is also effective for sterilizing breast milk, which extends beyond the 5-log reduction of pasteurization to an 8-log bacteria reduction.[12] The highly penetrating gamma rays are formed by the self disintegration of a Cobalt-60 source. Exposure to gamma irradiation is considered physical sterilization because contaminants such as bacteria are destroyed by breaking down bacterial DNA, inhibiting replication.[19] The American Medical Association, World Health Organization, and Food and Drug Administration have endorsed irradiated food as safe, which has led to over 40 countries, including the US, utilizing IR for everything from fruit to meat.[20] IR treatment with 2.5 Mrads of exposure was shown to completely sterilize breast milk samples of *Staphylococcus albus*, group B *streptococci*, and *E. coli*. However, compared to holder pasteurization, IR caused up to a 32% decrease in IgA concentration (g/L) and up to an 80% decrease in lactoferrin concentration (g/L), whereas holder pasteurization caused no significant drop in average concentrations of either.[21]

The objective of this study was to investigate the effects of these alternative food processing technologies on donor breast milk BSSL activity. The treatments tested have already been proven to provide microbiological safety, but their effects on milk enzyme activity remain mostly unexplored.

Methods

Pooling

A large pool of frozen donor breast milk was donated by Northwest Mothers Milk Bank. From that pool, 9 L was removed from a -20°C freezer and placed at room temperature for 2h followed by 48h in a 4°C refrigerator to thaw the milk. After the complete thaw, individual packs of milk were pooled together and allocated into triplicates for each treatment. Three 60 mL-samples were allocated for high pressure processing (HPP), irradiation (IR), low temperature long time (LTLT), and raw untreated. All allocated samples were then placed back into a -20°C freezer until treatments were carried out.

High pressure processing (HPP)

Three frozen, 60 mL samples were partially thawed at room temperature (RT) for 2 hours, placed in individual plastic bags, and vacuum-sealed. The samples were then placed on dry ice and shipped to Cornell University and processed in a *Hiperbaric* commercial scale high pressure processing unit. At the Cornell HPP facility, the packaged samples were submerged in water and subjected to 550 MPa of pressure for five minutes.[18] Samples were placed back on dry ice immediately following treatment, shipped back to OSU, and stored in a -20°C freezer until analysis.

Gamma-cell Irradiation (IR)

Three frozen, 60 mL samples were irradiated at the Oregon State University Radiation Center. The samples were exposed to 2.5 Mrads of gamma cell irradiation from a Cobalt-60 source, as this is the standard amount for complete sterilization.[19,21] The samples were kept frozen for the duration of the five-hour treatment and then placed back in a -20°C immediately following treatment completion.

Low temperature long time (LTLT)

Three frozen, 60 mL samples in glass bottles were placed at RT for 3 hours followed by 4°C for 24 hours to thaw. A water bath was heated to 63 °C, which remained constant throughout the treatment. The three samples were then placed in the water bath for 30 minutes. After 30 minutes, the samples were placed in an ice bath until the milk dropped to 4°C and then were placed back in the -20°C freezer.

BSSL Assay Methods

A spectrometric assay was used to determine the activity of BSSL in milk samples. 1.5 mL milk samples from each treatment group were thawed on ice for 30 minutes and diluted 100x. A buffer solution was created according to modified methods presented by Albro et al.[22] A mixture of 0.4 M Tris-HCl in 270 mL of water was heated to 37 °C. 116 mM of sodium cholate was dissolved in 70 mL of water and added to the mixture. The pH was adjusted to 8 using 6 M NaOH. 14 mM p-nitrophenyl myristate was dissolved in 20 mL of 2-methoxyethanol and added to the mixture. Finally, 210 mL of water was added to the mixture, the pH was adjusted back to 8 using 6M NaOH if needed, and the buffer was heated back to 37 °C.

33.3 µL of milk from each treatment triplicate was pipetted in duplicate into a 96-well plate. A multichannel pipette was used to add 83.3 µL of water followed by 83.3 µL of buffer to each well. Production of p-nitrophenol was measured by monitoring absorbance at 405 nm every 5 minutes for 30 minutes at a constant temperature (37°C).

In order to the determine relative concentration, a standard curve using p-nitrophenol was also run on the plate. 13.6 mg of p-nitrophenol was dissolved in 4 mL of 0.25 N NaOH to create a basic 250 mM p-nitrophenol solution. The use of a basic solution is essential for the deprotonation of p-nitrophenol, the final product of which strongly absorbs light at 405 nm. 20

µL of the 250 mM solution was added to 980 µL of water to create a 500 uM solution. The 500 µM solution was then serial diluted to 250 µM, 125 µM, 62.5 µM and 31.25 µM. A blank of pure water was also prepared. 33.3 µL of each dilution was pipetted into the 96-well plate along with the buffer and water in the same manner as the milk samples. The standard curve obtained from the resulting absorbances was then used to calculate the end concentration of p-nitrophenol in the milk samples.

Calculations

The absorbance data collected from the spectrometer was converted to concentration using the standard curve equation of y=0.0019x-0.0257, which had an R^2 value of .998. Each of the 6 absorbances from each of the treatment groups was individually converted to concentration for all 7 time points.

$$y\ (concentration) = \frac{(.1842+0.0257)}{0.0019} = 110.482\ \text{uM}$$

Equation 1: Conversion of Raw milk absorbance (.1842) at time 0 mins to concentration of p-nitrophenol.

Concentration (uM) was then converted to moles of p-nitrophenol/ L of milk in order to have a standard unit for comparisons. Once again, all 7 time points for each of the 6 concentrations for each treatment group were calculated individually.

$$M = \frac{110.482uM}{.33} * 1000 * \frac{1000}{1000000} = 334.79 \frac{moles\ p\ nitrophenol}{L\ milk}$$

Equation 2: Conversion of Raw milk p-nitrophenol concentration to moles p-nitrophenol/ L milk.

To create Figure 1, the molarity (mol/L) at time point zero was subtracted from the final molarities at each time point for all 6 replicates in each treatment group. These values, referred to as the corrected molarities, were averaged at each time point and graphed for each treatment group.

The activity was defined as moles of p-nitrophenol/ L milk produced per minute. To find this value, the uncorrected molarity at 10 minutes was subtracted from the uncorrected molarity at 0 minutes and divided by 10 to get M/min. This was done for all 6 treatment replicates of each group and averaged. Standard Error was also calculated using these values. Ten minutes was used as this was the upper limit of the standard curve. This process was then repeated at the 5 min time interval.

Statistics

One-Way ANOVA with repeated measures was conducted on calculated BSSL Activity (M/min) data for all treatment groups. This was followed by a Tukey's multiple comparisons test (RStudio, version 3.3.2) to individually compare BSSL Activity of all four treatment groups. This process was repeated for total BSSL Activity determined after 5 minutes of incubation time and 10 minutes of incubation time, as well as for total p-nitrophenol produced. Differences in BSSL Activity were designated significant at $p < .05$.

Results

In order to determine BSSL activity, absorbance collected from the spectrometric assay was converted to concentration of p-nitrophenyl myristate cleaved to p-nitrophenol by BSSL in each milk sample. This was done with the use of a standard curve of p-nitrophenol, with the calculations discussed in the methods section. The ability of the assay to detect continuous production of p-nitrophenol over the course of the 10-minute incubation time in the spectrometer was demonstrated to confirm the efficacy of the assay protocol (Figure 1). BSSL Activity data was calculated at both 5 and 10 minutes to determine a minimum assay run time. Results obtained from both run times provided identical results in regards to significant activity difference between groups ($p < .005$, Figures 2 and 3).

A significant difference in the amount of p-nitrophenyl myristate cleaved by BSSL was found between all of the treatment groups ($p < .005$, Figure 1). The highest concentration of p-nitrophenol reached in LTLT treated milk was 54.33 M, in IR treated milk was 305.68 M, in HPP treated was 801.46M, and in raw untreated was 957.94M.

The final BSSL activities for each treatment type are shown in Figure 2. The activity was defined as moles of p-nitrophenol produced per L of milk per minute and was calculated according to the calculations described above. Raw untreated milk had the highest activity with an average activity of 95.74 M/min, followed by HPP treated mil with 80.16 M/min, IR treated milk with 30.56 M/min, and finally LTLT treated milk with 5.43 M/min.

Figure 1

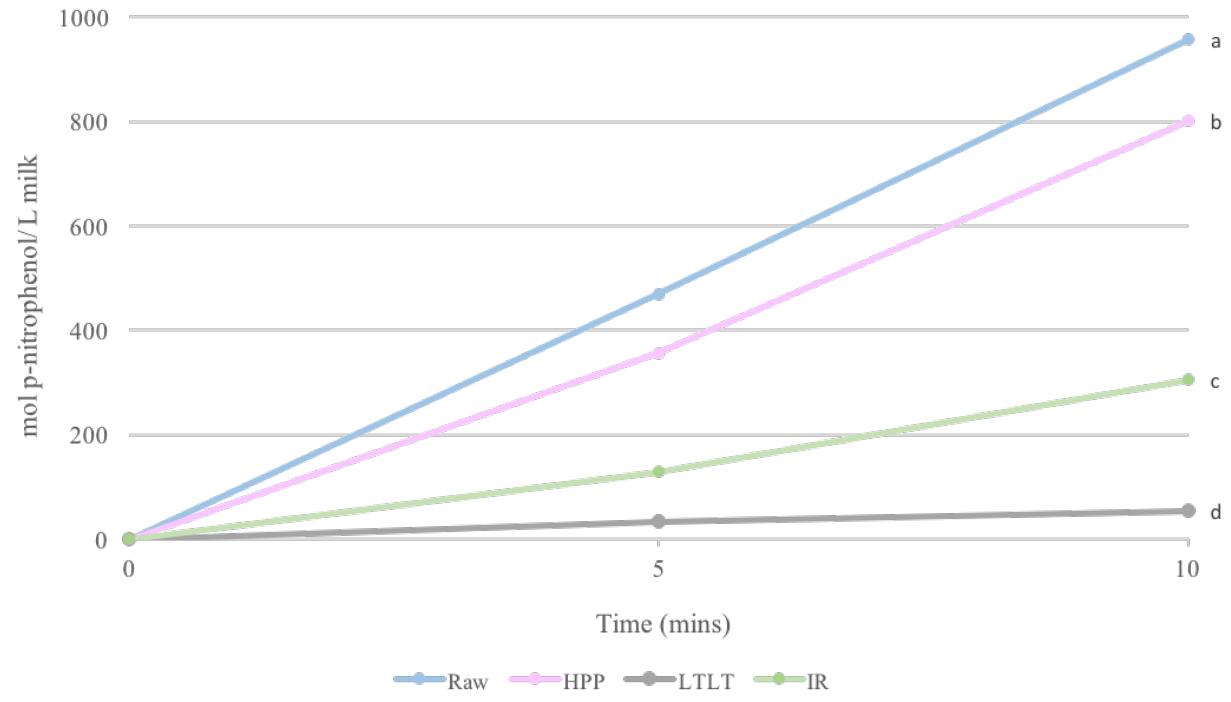

Figure 2

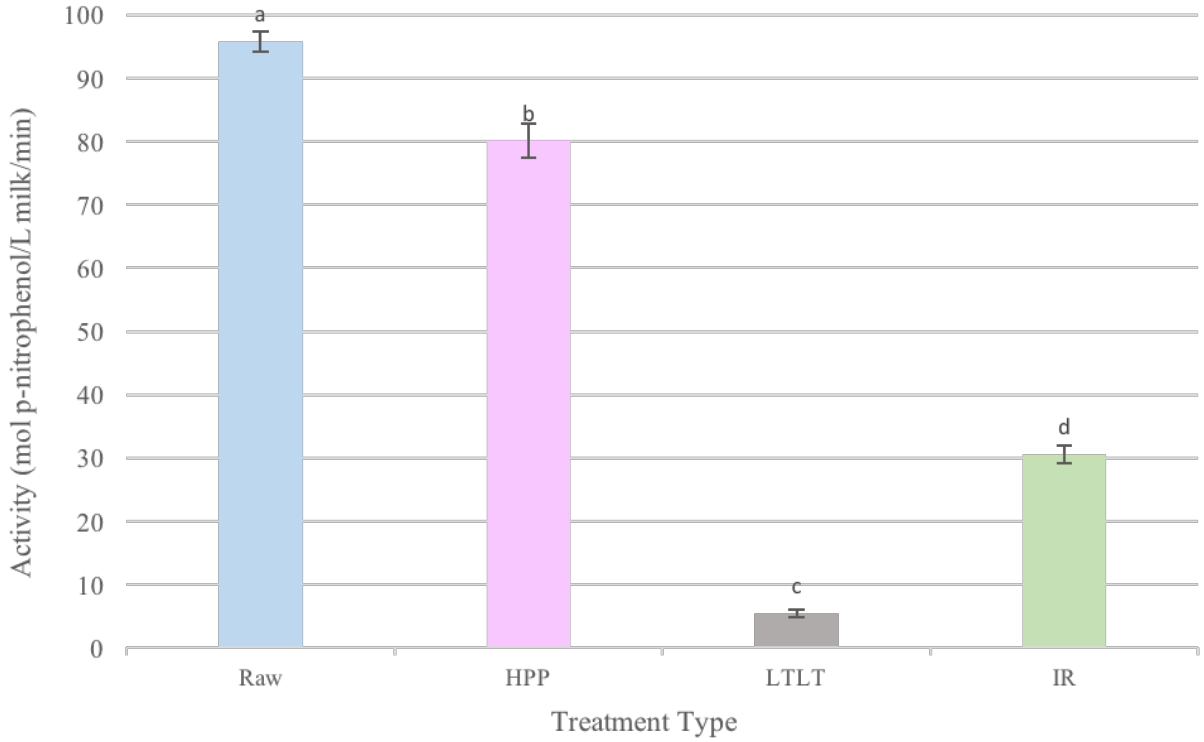

Figure 3

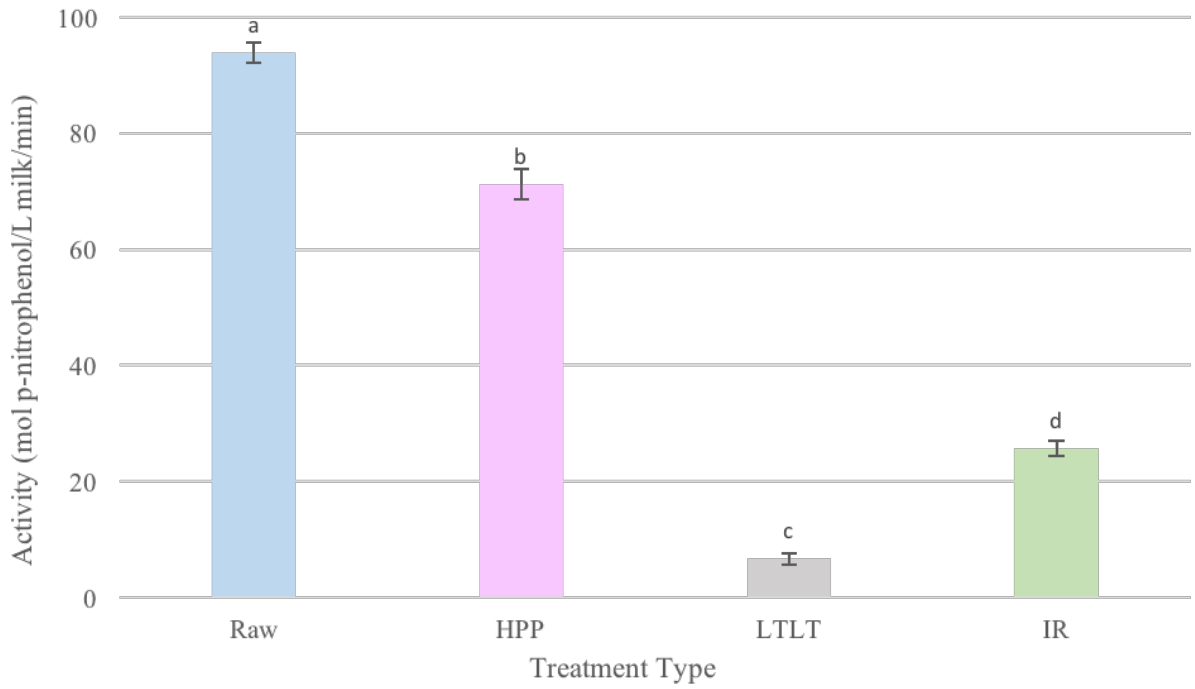

Figure Legend

Figure 1: The amount of p-nitrophenol (mol/L of milk) produced by cleavage of p-nitrophenyl myristate by BSSL over the 10-minute incubation period by each treatment group. Between-group significance represented by different lettering (a, b, c, d).
Figure 2 and 3: The amount of p-nitrophenol (mol/L of milk) produced by cleavage of p-nitrophenyl myristate by BSSL per minute for 10 minutes and 5 minutes, respectively, by each treatment group. Standard error within treatment groups is represented by the error bars. Between-group significance represented by differing letters (a, b, c, d).

Discussion

Treatment Impact on BSSL

Fat digestion is essential to the growth of neonatal infants and bile salt-stimulated lipase plays an important role in the breakdown of lipids. As mothers of neonates often cannot produce adequate breast milk supplies, these infants are often fed donor breast milk proceeded by milk banks. Due to its ease and ability to ensure the microbiological safety of this donor milk, holder pasteurization (62.5 ‘C, 30 mins) is the current standard for milk banks. However, this treatment was shown to decrease BSSL activity by 95% when compared to raw, untreated milk (Figure 1). Even after 10 minutes of optimal conditions and substrate concentration, LTLT treated milk produced 96% less p-nitrophenol than raw, untreated milk. As the p-nitrophenol was meant to mimic the fatty acid tails cleaved off of lipids by BSSL, these results demonstrate the significant inactivation of BSSL by LTLT treatment. The decreased activity and overall amount of p-nitrophenol produced indicates that neonates given milk treated this way have a decreased ability to digest fat, a key nutrient for growth. Previous studies have shown that the implication of this decrease in BSSL activity are reduced weight gain and linear growth.[1,6,7] Therefore, the necessity of an alternative treatment is even more apparent.

Gamma cell irradiation (IR) was tested as a potential alternative due to its ability to completely sterilize milk samples.[21] While significantly more activity was observed when compared to LTLT treated milk ($p<.005$), a 68% reduction in activity and a 44% reduction in p-nitrophenol production was also observed, when compared to raw, untreated milk (Figures 1 and 2). The increase of BSSL activity over LTLT treated milk could make it a candidate for an alternative treatment, but the overall feasibility for milk banks decreases that likelihood. Complete cobalt-60 irradiation systems cost upwards of $3 million dollars and require 10X

longer treatment times than LTLT.[20] Current procedures for LTLT treatment requires only a consistently heated water bath, so an investment in a gamma cell irradiator would be a significant one for a milk bank. Should they choose to use an Irradiation Service Center instead, there would be the additional cost and time of shipping pre-packaged milk to and from the service centers. That being said, the use of an external site could decrease labor costs for the milk bank as well reach a higher reduction of bacterial contaminants, which would be ideal for ensuring the safety of donor milk. Overall, the fact that there is still a substantial decrease in BSSL activity is the greatest cause of concerns over the worth of this alternative treatment (Figure 2).

High pressure processing (HPP) was shown to preserve the most amount of BSSL activity when compared to LTLT and IR. Only a 16% drop is activity levels and an 8% reduction in p-nitrophenol production compared to raw, untreated milk was observed (Figures 1 and 2). While this reduction was significant ($p<.005$), the increase in activity and p-nitrophenol production when compared to LTLT and IR was as well ($p<.005$). Of the three treatments tested, HPP was shown to have BSSL activity most comparable to raw, untreated milk. The ability of this treatment to also kill important breast milk pathogens makes it an ideal candidate to replace LTLT treatment. However, complications with this treatment also lie in the cost associated with the necessary equipment. A 55L HPP system, which is suitable for small to medium sized companies, can cost upwards of $700,000 to purchase and install.[23] Similar to IR, the use of this treatment would most likely require the shipping of milk to centers with established HPP systems, with the same cost and benefits that were discussed with IR. While the cost analysis does decrease the feasibility of HPP, the significant retention of BSSL activity has the potential to greatly improve the fat digestion and growth of preterm infants, making it a worthwhile alternative to explore further. Due to the wide range of possible treatment parameters possible,

future studies should focus on finding the lowest pressure and time that ensures both microbiological safety and retention of BSSL and other important bioactive proteins.

BSSL Activity Assay

BSSL Activity was measured used a modified assay first developed by Albro et al.[22] The molarities of each component in the buffer solution were kept consistent as described by Albro, but the order in which they were assembled needed additional detail. Due to the hydrophobic nature of the p-nitrophenyl myristate, the substrate was difficult to dissolve in the water-based buffer. Three modifications were made to keep the substrate in solution and ensure that an equal concentration of p-nitrophenol myristate was placed in each well with the milk samples. The first was the initial dissolving of the p-nitrophenol myristate in the 2-methoxyethanol. The substrate was dissolved separately in the 2-methoxyethanol before being placed in the heated buffer solution, which was shown to improve dissolution when compared to placing the p-nitrophenyl myristate in the solution undissolved. In the final solution, all of the substrate was completely dissolved. The second was the adaptation of the solution for a 96-well plate instead of a cuvette. Instead of the 5 mL of the buffer used by Albro, 166.67 uL was used in each well. Finally, due to the fact that the buffer turns a light yellow color, initial spectrometer readings with the original concentration were inconclusive and showed no significant difference in absorbance between blanks and samples wells. In order to decrease the color interference, the buffer was 1:2 diluted in the wells. This dilution had to be done in each well individually to prevent the added water from pulling the p-nitrophenyl out of solution. With the dilution, the color of the buffer became clear and the results discussed above were obtained.

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